Standard Operation Procedures (SOPs) and Chain Of Custody (COC) Format

Appendix 3.4

Standard Operation Procedures (SOPs) and Chain Of Custody (COC) Format

for

Efficacy Test

Appendix 3.4.1

SOP: Analysis of Organism and their Viability in the Ballast Water

(Organism of ≥10-50 μm dimension)

KOMERI SOP BWMS 04

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Busan, Rep. of Korea

Prepared by Jeong-Kyeong Park
Approved by Young-Soo Kim
December 2009
Revision 3.0

.1. Scopes and Application

This method describes a viable algal staining and re-growth procedure for the assessment of ship's ballast water treatment system (BWTS).

.2. Equipment, Reagent and Supplies

- Inverted fluorescent microscope (×400 magnification)
- 10, 20 and 50 µm nylon mesh
- Gridded counting plate (Sedgewick Rafter chamber and Neubauer Improved counting chamber)
- Mass cylinder, beaker
- 5-CFDA-AM (Appendix 1)
- Micro pipette, 96 well plate, f/2-medium(Appendix 2), incubator

.3. Sampling and preparation

- Sampling the treated (10 L) and/or control (1 L) water in ballast water tank


- Sample concentrate by 50 µm, 10 µm nylon mesh

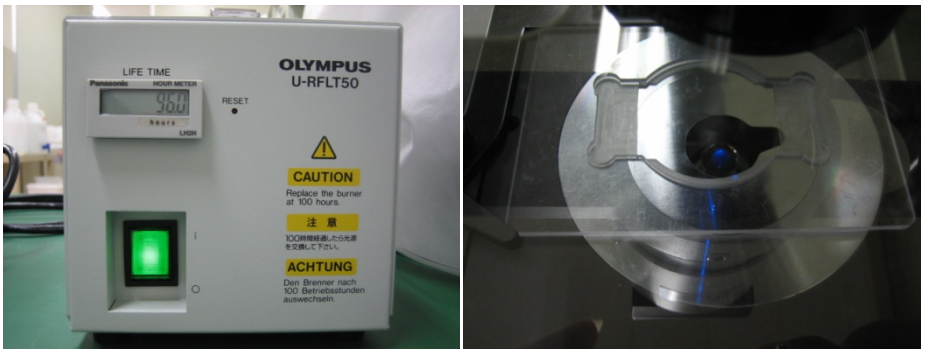
.4. Sample analysis (procedure)

(1) After mixing the sample, 1 mL of sample place on a counting chamber.

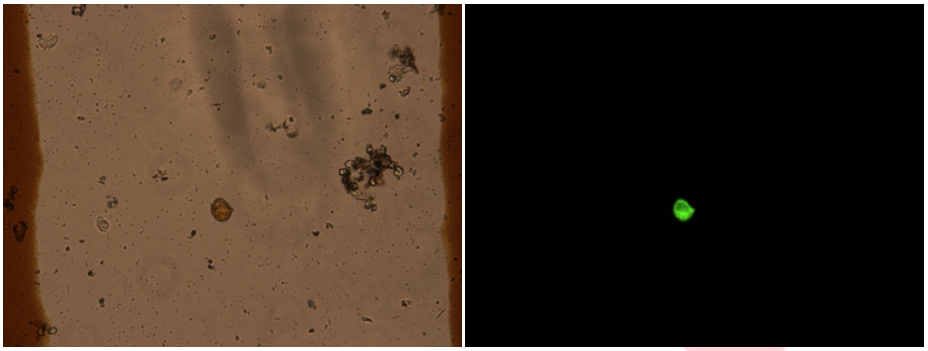
(2) Add > 1 µL of 5-CFDA-AM working solution to a counting chamber.

(3) Wait for 30 minutes for cell staining.

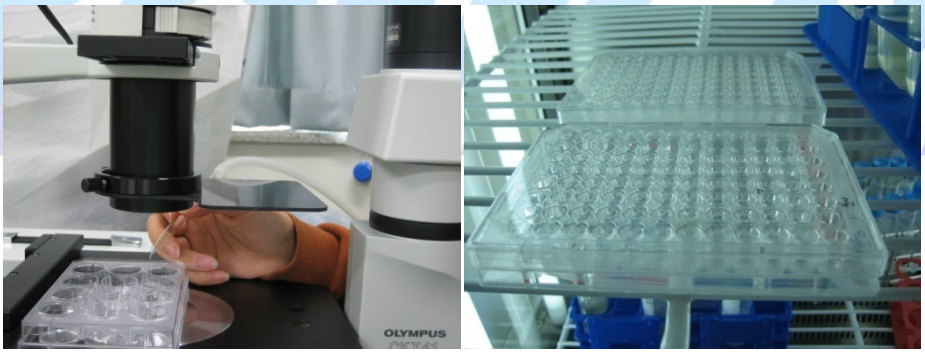
(4) Turn on mercury burner of microscope and apply to a fluorescent filter (Blue light).



(5) Count fluorescent unit (Viable cells represent **green color**).



(6) Isolate viable organism in treated water by a micro pipette, and incubate in f/2 medium under 50 μ mol s^{-1} cm^{-2} at same water temperature with field condition during ten days.



Appendix 1

5-CFDA-AM

1. Making of 5-CFDA-AM Stock Solution (4 mM)
   100% DMSO solution inject to a vial of 5-CFDA-AM (M.W. 532.46 g), which keep frozen until use.
   (CFDA –AM: Ex. = 530 nm, Em. = 541 nm)
2. Working Solution
   Add > 1 μL of 5-CFDA-AM stock solution to a 1 mL of sample (final concentration is 4 μM)

Appendix 2

f/2 medium

1. Guillard's (f/2) Marine water enrichment solution, 50x (sigma G9903)
   Stock solution 20 mL dilute with 1 L filtered sea water

Compound	Stock solution	Quantity
$NaNO_3$	75.0 g/L dH_2O	1.0 mL
$NaH_2PO{\cdot}4H_2O$	5.0 g/L dH_2O	1.0 mL
$Na_2SiO_3{\cdot}9H_2O$	30.0 g/L dH_2O	2.0 mL
f/2 Trace metal solution	(see recipe below)	1.0 mL
f/2 Vitamin solution	(see recipe below)	0.5 mL

f/2 Trace metal solution (Guillard & Ryther 1962, Guillard 1975)

Compound	Stock solution	Quantity
$FeCl_3{\cdot}6H_2O$	-	3.15 g
$Na_2EDTA{\cdot}2H_2O$	-	4.36 g
$CuSO_4{\cdot}5H_2O$	9.8 g/L dH_2O	1.0 mL
$Na_2MoO_4{\cdot}2H_2O$	6.3 g/L dH_2O	1.0 mL
$ZnSO_4{\cdot}7H2O$	22.0 g/L dH_2O	1.0 mL
$CoCl_2{\cdot}6H_2O$	10.0 g/L dH_2O	1.0 mL
$MnCl_2{\cdot}4H_2O$	180.0 g/L dH_2O	1.0 mL

f/2 Vitamin solution (Guillard & Ryther 1962, Guillard 1975)

Compound	Stock solution	Quantity
Vitamin B_{12}	1.0 g/L dH_2O	1.0 mL
Biotin	0.1 g/L dH_2O	10.0 mL
Thiamin HCl	-	200.0 mg

SOP: Analysis of Organism and their Viability in the Ballast Water

(Organism of ≥ 50 μm dimension)

KOMERI SOP BWMS 06

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Prepared by Eun-Ju Seong
Approved by Young-Soo Kim
April 2011
Revision 4.0

.1. Scopes and Application

This method describes ≥ 50 μm viable organism behavior procedure for the assessment of ship's ballast water treatment system (BWTS).

.2. Equipment and Supplies

- 50 μm nylon mesh
- Bogorov counting chamber
- Bottle (1 L-HDPE)
- Conical tube (50 mL)
- Counter
- Mass cylinder, beaker
- Plankton net (50 μm mesh)
- Sealed Pasteur-pipette (145 mm)
- Micro-pipette (1000 μL)
- Stereo microscope (×40 magnification)

.3. Reagent and Standards

- Stock solution : 1% Neutral red solution (Neutral red 1 g / 99 g in de-ionized water)
- Working solution : 0.05 % Neutral red solution (1% Neutral red solution 5 mL / 95 mL in de-ionized water)
  (ACROS ORGANICS Inc., New Jersey USA, C553-24-2, Lot. No. 041101)
- Acidic solution : Glacial acetic acid

.4. Procedure

(1) Sampling the treated (1 m^3) and/or control (20 L) water in ballast water tank.

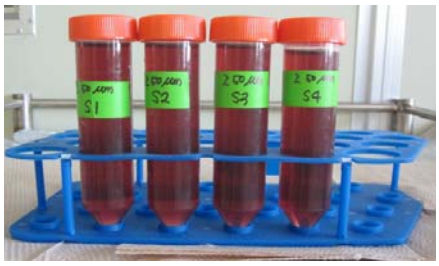

(2) Sample concentrates by 50 μm nylon mesh.

(3) Add more than 20 mL of 0.05% neutral red solution into concentrated sample bottle (1 L).

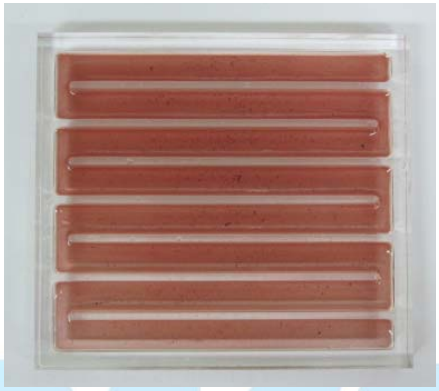

(4) Inject the glacial acetic acid solution if sample is over the pH 8.0.

(5) Wait a minimum of one hour for individuals staining.

(6) Sample concentrates by a 50 μm nylon mesh to final volume 50 mL.



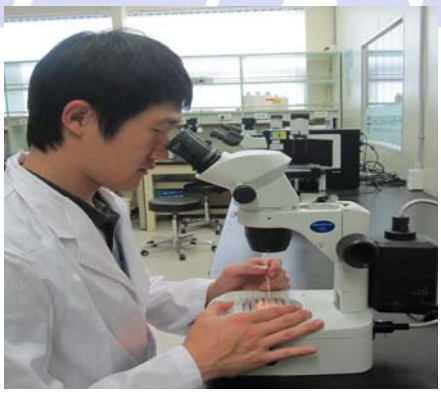
(7) 10 mL of concentrated sub-sample place on a Bogorov counting chamber.



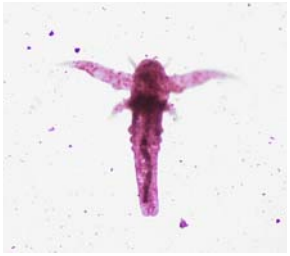
(8) Turn on the microscope and gently touched with a sealed Pasteur-pipette (A). Only using the touch method (B).

A)

B)



(9) Count stained and moving individuals (Viable individuals represent red color).



SOP: Plate Count for Heterotrophic Bacteria

KOMERI SOP BWMS 10

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Approved by Young-Soo Kim
April 2011
Revision 4.0

.1. Scopes and Application

This heterotrophic plate count (HPC) is a procedure for estimating the number of live heterotrophic bacteria in ballast water. Colonies may arise from pairs, chains, clusters, or single cells, all of which are included in the term “Colony-Forming Units” (CFU).

.2. Equipment and Supplies

- Incubator maintained (15 ± 0.5~ 50 ± 0.5) ^{o}C
- Hand tally or electrical counting device
- Pipette, Flask
- Thermometer
- Petri dish, sterile, plastic, 90 × 15 mm
- Water bath maintained at 50 ^{o}C for tempering agar
- Test tubes, screw cap, 16 × 125 mm, borosilicate glass or plastic
- Glass rod, 4 mm diameter, fire polished end, 200 mm in length, bend 45° about 40 mm from one end

.3. Reagent and Standards

Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The agar used in preparation of culture media must be of microbiological grade.

(1) ***R2A agar***
(2) ***NWRI agar***

.4. Procedure

(1) Pipette 0.1 to 0.5 mL sample onto surface of pre-dried agar plate (If necessary, a sample can be dilution) (Figure A).
(2) Using a sterile bent glass rod, distribute inoculum over surface on the medium by rotating the dish by hand or on a turntable (Figure B).
(3) Let inoculum be absorbed completely into the medium before incubating.
(4) Incubate at (Figure C)

R2A agar	NWRI agar
28 ^{o}C, 7 days	20 ^{o}C, 7 days

(5) Count all colonies on selected plates promptly after incubation (Figure D).

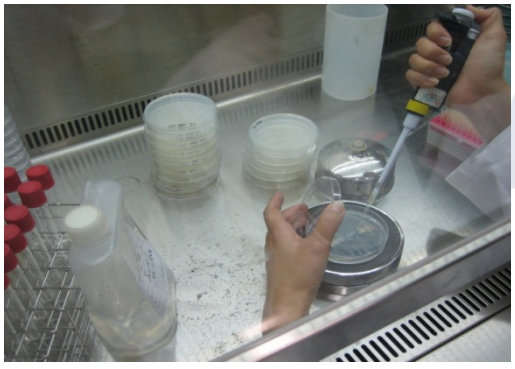
Figure A


Figure B

Figure C

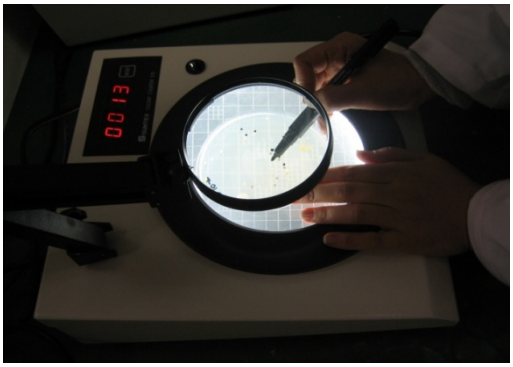

Figure D

.5. Data Analysis and Calculations

Use the following general rules to calculate the heterotrophic plate bacteria count per mL of sample:

(1) Select the plates having 30 to 300 colonies in determining the plate count. Compute bacterial count per mL by the following equation:

$$\text{Heterotrophic bacteria (cells) / mL} = \frac{\text{Colonies counted}}{\text{Actual volume of sample in dish, mL}}$$

SOP: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (Modified mTEC)

KOMERI SOP BWMS 11

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Prepared by Soo-Yeon Im
Approved by Young-Soo Kim
April 2011
Revision 4.0

.1. Scopes and Application

This method describes a revised *Escherichia coli* membrane filter (MF) procedure, a single-step method that uses one medium, modified mTEC Agar, and does not require the transfer of the membrane filter to another medium or other substrate. The modified medium contains a chromogen (5-bromo-6-chloro-3-indolyl-β-D-glucuronide), which is catabolized to glucuronic acid and a red or magenta colored compound by *E. coli* that produce the enzyme β-D-glucuronidase.

.2. Equipment and Supplies

- Glass lens with magnification of 2-5 x, or stereoscopic microscope
- Hand tally or electronic counting device
- Pipettes, stainless steel, aluminum or borosilicate glass, for glass pipettes
- Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source
- Filter flask, vacuum, usually 1 L, with appropriate tubing
- Flask for safety trap placed between the filter flask and the vacuum source
- Forceps, straight or curved, with smooth tips to handle filters without damage
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles
- Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23
- Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids; or 15 x 60 mm, glass
- Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10dilutions
- Flasks, borosilicate glass, screw-cap, 250-2000 mL volume
- Membrane filters, sterile, white, grid marked, 47 mm diameter, with (0.45 ± 0.02) µm pore size
- Inoculation loops, at least 3 mm diameter, and needles, platinum wire, 26 B & S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops. (Note: A platinum loop is required for the cytochrome oxidase test in the verification procedure.)
- Incubator maintained at (35 ± 0.5) °C, with approximately 90 % humidity if loose-lidded petri dishes are used
- Water-bath maintained at (44.5 ± 0.5) °C
- Water-bath maintained at 50 °C for tempering agar
- Test tubes, 20 x 150 mm, 10 x 75 mm, borosilicate glass or plastic
- Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes
- Test tubes screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size

.3. Reagent and Standards

Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control.

Purity of Water: Reagent water conforming to Specification D1193, reagent water conforming Type II, Annual Book of ASTM Standards.

(1) ***Phosphate Buffered Saline***
(2) ***Phosphate Buffered Dilution Water***
(3) ***Preparation of Stock Magnesium Chloride Solution:***
(4) ***Working Phosphate Buffered Dilution Water:***
(5) ***Modified mTEC agar (Difco 214884 or 214880)***
(6) ***Nutrient Agar (Difco 0001, BD 4311472)***

(7) ***Tryptic Soy Broth (Difco 0370); Trypticase Soy Broth (BD 99071)***
(8) ***Simmons Citrate Agar (Difco 0091, BD 4311620)***
(9) ***Tryptone 1 % (Difco 0123); Tryptophane Broth (BD 4321717 and 4321718)***
(10) ***EC Broth (Difco 0314, BD 4311187)***
(11) ***Oxidase Reagent***
(12) ***Kovacs Indole Reagent***

.4. Procedure

(1) Prepare the modified mTEC Agar as directed in section 3.
(2) Mark the petri dish and report form with the sample identification and volume.
(3) Place a sterile membrane filter on the filter base, grid side up, and attach the funnel to the base so that the membrane filter is held between the funnel and the base (Figure A).
(4) Shake the sample bottle vigorously at least 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel (Figure B).
(5) Select sample volumes based on previous knowledge of the pollution level, to produce 20-80 *E. coli* colonies on the membranes.
(6) Smaller sample sizes or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions may be filtered.
(7) Filter the sample, and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum, and remove the funnel from the filter base (Figure C).
(8) Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the modified mTEC Agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate (35 ± 0.5) °C for 2 h (Figure D).
(9) After 2 h incubation at (35 ± 0.5) °C, transfer the plate to a Whirl-Pak® bag, seal the bag, place the bag with the plate inverted in a test-tube rack, and put the rack in a (44.5 ± 0.2) °C water bath for 22-24 h (Figure E).
(10) After 22-24 h, remove the plate from the water bath, count and record the number of red or magenta colonies with the aid of an illuminated lens with a 2-5 x magnification or a stereoscopic microscope (Figure F).

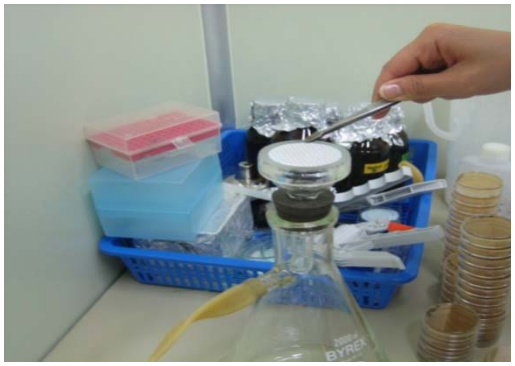
Figure A

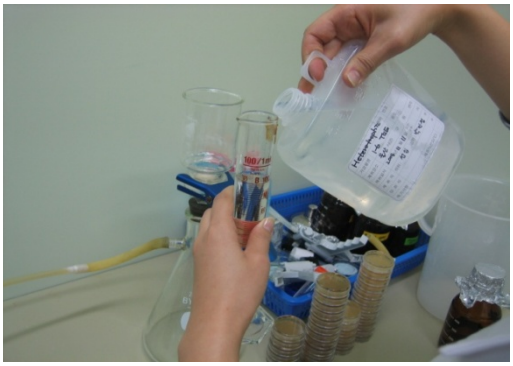
Figure B

Figure C

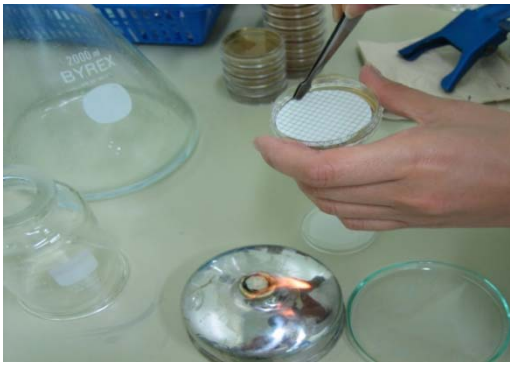

Figure D

Figure E

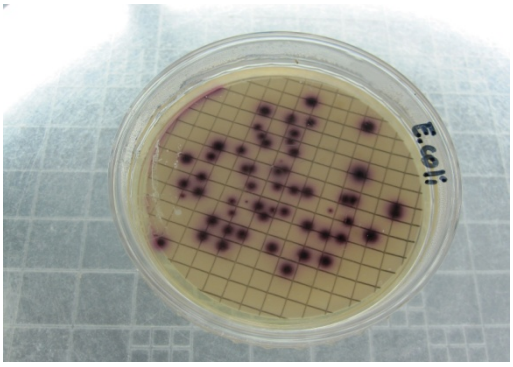

Figure F

.5. Data Analysis and Calculations

Use the following general rules to calculate the *E. coli* count per 100 mL of sample:

Select the membrane filter with an acceptable number of magenta or red colonies (20-80), and calculate the number of *E. coli* per 100 mL according to the following general formula:

$$E.\ coli\ /\ 100\ mL = \frac{\text{Number of } E.\ coli \text{ colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

.6. Verification Procedure

Red or magenta colonies can be verified as *E. coli*. Verification of colonies may be required in evidence gathering and is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

(1) Using a sterile inoculation loop, transfer growth from the centers of at least 10 well-isolated colonies to Nutrient Agar plates or slants and to Trypticase Soy Broth. Incubate the agar and broth cultures for 24 h at (35 ± 0.5) °C.

(2) After incubation, remove a loopful of growth from the Nutrient Agar slant with a platinum loop, and deposit it on the surface of a piece of filter paper that has been saturated with freshly prepared Cytochrome Oxidase Reagent. If the spot where the bacteria were deposited turns deep purple within 15 seconds, the test is positive.

(3) Transfer growth from the Trypticase Soy Broth tube to Simmons Citrate Agar, Tryptone Broth, and an EC Broth fermentation tube.

1) Incubate the Simmons Citrate Agar and Tryptone Broth for 48 h at (35 ± 0.5) °C.

2) Incubate the EC Broth at (44.5 ± 0.2) °C in a waterbath for 24 h. The water level must be above the level of the EC Broth in the tube.

3) Add 0.5 mL of Kovacs Indole Reagent to the 48 h Tryptone Broth culture, and shake the tube gently. A positive test for indole is indicated by a deep red color which develops in the alcohol layer on top of the broth.
4) *E. coli* is EC gas-positive, indole-positive, and oxidase-nagative, and does not utilize citrate (i.e., the medium remains green).

SOP: Enterococci in water by membrane filtration using membrane-Enterococcus indoxyl-β-D-glucoside agar (mEI)

KOMERI SOP BWMS 12

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April 2011
Revision 4.0

.1. Scopes and Application

This method describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. Enterococci are commonly found in the feces of humans and other warm-blooded animals. The enterococci test measures the bacteriological quality of recreational water.

.2. Equipment and Supplies

- Glass lens with magnification of 2-5 x or stereoscopic microscope
- Hand tally or electronic counting device
- Pipettes, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume
- Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil
- Ultraviolet unit for sanitization of the filter funnel between filtrations (optional)
- Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used
- Flask, filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional
- Flask for safety trap placed between the filter flask and the vacuum source
- Forceps, straight or curved, with smooth tips to handle filters without damage
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles
- Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23
- Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids
- Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions
- Flasks, borosilicate glass, screw-cap, 250-2000 mL volume
- Membrane filters, sterile, white, grid marked, 47 mm diameter, with (0.45 ± 0.02) µm pore size
- Inoculation loops, at least 3 mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops.
- Incubator maintained at (41 ± 0.5) °C
- Waterbath maintained at 50 °C for tempering agar
- Test tubes, 20 x 150 mm, borosilicate glass or plastic
- Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes
- Test tubes, screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size

.3. Reagent and Standards

Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control. Purity of Water: Reagent water conforming to Specification D1193, reagent water conforming Type II, Annual Book of ASTM Standards.

(1) ***Buffered Dilution Water***
(2) ***Phosphate Buffered Dilution Water***
(3) ***Stock Phosphate Buffer Solution***
(4) ***mEI Agar***

(5) ***Brain Heart Infusion Broth (BHIB) (Difco 0037, BD 4311059)***
(6) ***Brain Heart Infusion Broth (BHIB) with 6.5% NaC1***
(7) ***Brain Heart Infusion Agar (BHIA) (Difco 0418, BD4311065)***
(8) ***Bile Esculin Agar (BEA) (Difco 0879)***

.4. Procedure

(1) Prepare the mEI agar as directed in section 3.
(2) Mark the petri dishes and report forms with sample identification and sample volumes.
(3) Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base so that the membrane filter is now held between the funnel and the base (Figure A).
(4) Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel (Figure B).
(5) Select sample volumes based on previous knowledge of the pollution level, to produce 20-60 *enterococci* colonies on membranes.
(6) Smaller sample size or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions may be filtered, and the results may be combined.
(7) Filter the sample, and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base (Figure C).
(8) Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the mEI agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at (41 ± 0.5) °C for 24 h (Figure D & E).
(9) After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 colonies with a blue halo regardless of colony color as an enterococci (Figure F). Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

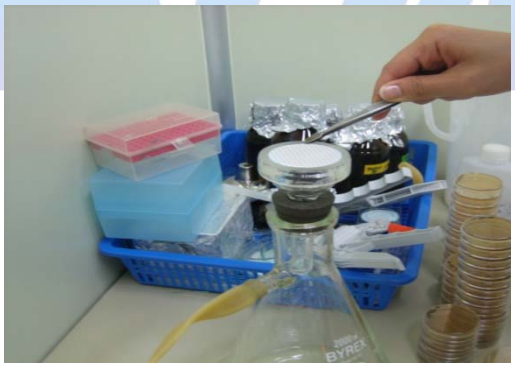

Figure A

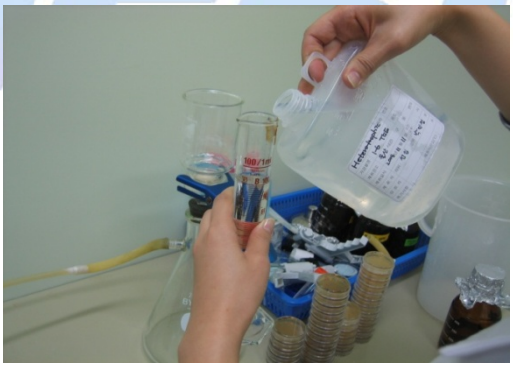
Figure B

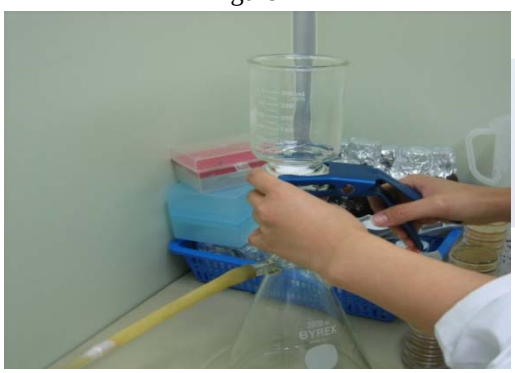

Figure C

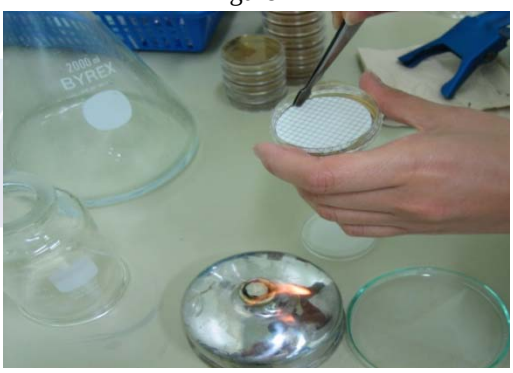

Figure D

Figure E

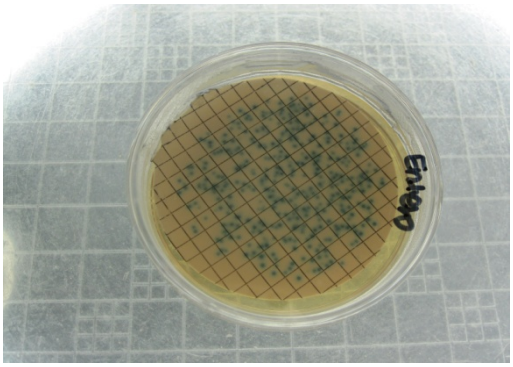

Figure F

.5. Data Analysis and Calculations

Use the following general rules to calculate the enterococci count per 100 mL of sample:

Select the membrane filter with an acceptable number of colonies (regardless of colony color) with a blue halo (20-60). Calculate the number of enterococci per 100 mL according to the following general formula:

$$\text{Enterococci / 100 mL} = \frac{\text{Number of enterococci colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

.6. Verification Procedure

Colonies of any color having a blue halo after incubation on mEI agar can be verified as enterococci. Verification of colonies may be required in evidence gathering and it is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

(1) Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a BHIB tube and onto a BHIA slant. Incubate broth tubes for 24 h and slants for 48 h at (35 ± 0.5) °C.
(2) After 24 h incubation, transfer a loopful of material from each BHIB tube to BEA, BHIB and BHIB with 6.5 % NaCl.
    1) Incubate the BEA and BHIB with 6.5% NaCl at (35 ± 0.5) °C for 48 h.
    2) Incubate the BHIB at (45 ± 0.5) °C for 48 h.
(3) Observe for growth.
(4) After 48 h incubation, apply a Gram stain to growth from each BHIA slant.
(5) Gram-positive enterococci that grow and hydrolyze esculin on BEA (*i.e.*, produce a black or brown precipitate), and grow in BHIB at (45 ± 0.5) °C and BHIB with 6.5 % NaCl at (35 ± 0.5) °C are verified as enterococci.

SOP: Detection and viability of *Vibrio cholerae* in water by Standard Methods 9260 H

KOMERI SOP BWMS 13

Korea Marine Equipment Research Institute
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1631-10 Songjeong-dong Gangseo-gu
Busan, Rep. of Korea

Prepared by Soo-Yeon Im
Approved by Young-Soo Kim
April 2011
Revision 4.0

.1. Scopes and Application

This method should be used for detection and viability assessment of *Vibrio cholerae* in solid, particulate, liquid, and water samples.

.2. Equipment and Supplies

- Glass lens with magnification of 2-5 x, or stereoscopic microscope
- Hand tally or electronic counting device
- Ultraviolet unit for sanitization of the filter funnel between filtrations (optional)
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles
- Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23
- Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids; or 15 x 60 mm, glass or plastic, with loose-fitting lids; or 15 x 100 mm
- Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10dilutions
- Flasks, borosilicate glass, screw-cap, 250-2000 mL volume
- Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops.
- Incubator maintained at 37 °C, with approximately 90% humidity if loose-lidded petri dishes are used
- Test tubes, 20 x 150 mm, borosilicate glass or plastic
- Test tubes, 10 x 75 mm, borosilicate glass
- Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes
- Test tubes screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size

.3. Reagent and Standards

Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control.

(1) ***Alkaline peptone water (APW)***
(2) ***Thiosulfate Citrate Bile Salts Sucrose(TCBS) Agar (Difco 221872)***
(3) ***Tryptic Soy Agar (Difco 236950)***
(4) ***Simmons Citrate Agar (Difco 0091, BD 4311620)***
(5) ***Tryptone 1% (Difco 0123); Tryptophane Broth (BD 4321717 and 4321718)***

(6) ***Oxidase Reagent***

(7) ***Kovacs Indole Reagent***

(8) ***MR-VP Medium (Difco 216300)***

(9) ***Methyl Red Reagent***

(10) ***Kovacs Indole Reagent***

(11) ***Voges-Proskauer Reagent (VP-1)***

(12) ***Voges-Proskauer Reagent (VP-2)***

.4. Procedure

(1) Samples are enriched in alkaline peptone broth (APW) and incubated for up to 8 h.

(2) Thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates are inoculated with the incubated broth (Figure A) and incubated for 24 h (Figure B).

(3) Yellow sucrose-fermenting colonies are presumptive for *Vibrio cholerae* (Figure C) and are placed on tryptic soy agar with 0.5% NaCl.

(4) Presumptive positive colonies are then subjected to biochemical characterization by IMViC test (Indole, Methyl red, Voges-Proskauer, Citrate).

- Indole test : Inoculate tubes of tryptone broth and incubate at 35-37 °C for 22-36 h. Add 0.2-0.3 mL of Kovac's reagent. A positive test for indole is indicated by the appearance of a deep-red color within 5 min in the upper layer.
- Voges-Proskauer test : Inoculate tubes of 5 mL MR-VP broth and incubate at 35-37 °C for 46-50 h. Transfer 1 mL to tube and add 0.6 mL of VP-1 solution and 0.2 mL of 40% KOH (VP-2) and shake. Add a few crystal of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops, indicating the presence of acetylmethylcarbinol.
- Mehtyl red test : Incubate MR-VP tubes for additional 40-50 h at 35-37 °C after Voges-Proskauer test. Add 5 drops methyl red solution to each tube. A distinct red color indicates the presence of acid and is recorded as positive. Yellow is a negative reaction.
- Citrate test : Using an inoculating needle, lightly inoculate Simmons Citrate agar and incubate for 24-48 h at 35-37 °C in aerobic atmosphere. A positive reaction is indicated by growth with an intense blue color in the slant.
- Interpretation : All cultures indicating IMViC patterns ++(+/-)+ are considered to be *V. cholerae* (Figure D).

(5) *V. cholerae* will grow in nutrient broth without added NaCl. Confirmation is performed using slide agglutination assays by O1 and O139 antiserum for serological identification.

(6) A commercially available API 20E (BioMerieux, Inc.) kit used for the identification of *V. cholerae.* A plastic strip holding twenty mini-test tubes is inoculated with a saline suspension of a pure culture. After incubation in a humidity chamber for 18-24 h at 37 °C, the color reactions are read, and the reactions are converted to a seven-digit code (Figure E).

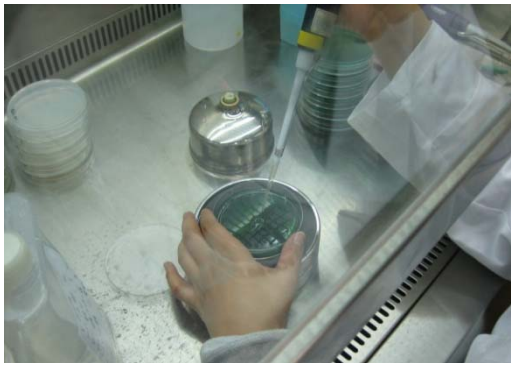

Figure A.

Figure B

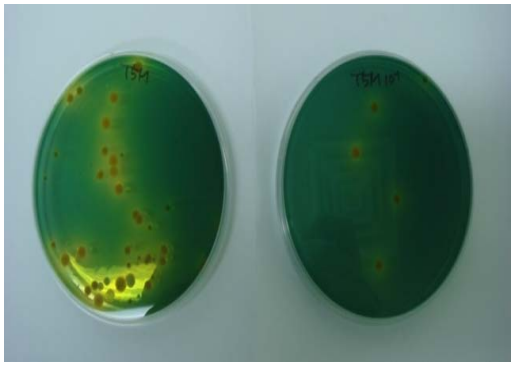

Figure C

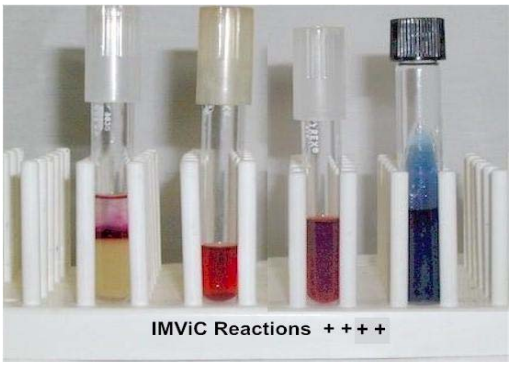

Figure D

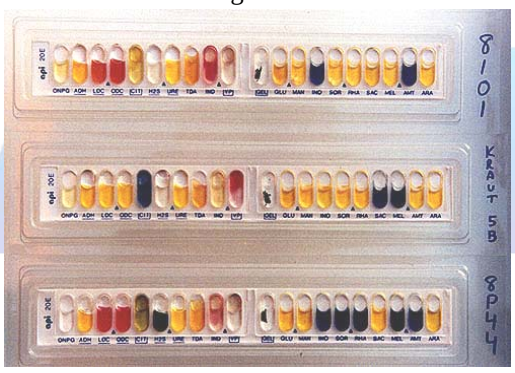

Figure E

.5. Data Analysis and Calculations

Calculate the number of *V. cholerae* O1 and O139 count per 100 mL of sample.

SOP: Total Suspended Solids in Water

KOMERI SOP BWMS 15

Korea Marine Equipment Research Institute
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1631-10 Songjeong-dong Gangseo-gu
Busan, Rep. of Korea

Prepared by Jun-Hak Lee
Approved by Young-Soo Kim
December 2009
Revision 3.0

.1 General

A well-mixed sample is evaporated in a weighted dish and dried to constant weight in an oven at 103 °C to 105 °C.

.2 Equipment and Supplies

- Drying oven
- GF/F filters, glass microfiber filters, white, 47 mm diameter
- Analytical balance, capable of weighing to 0.1 mg.
- Graduate cylinder
- Low-form beaker
- Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source.
- Filter flask, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional
- Flask for safety trap placed between the filter flask and the vacuum source
- Forceps, straight or curved, with smooth tips to handle filters without damage
- Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size

.3 Procedures

(1) Preparation of GF/F filters
- heat clean dish to 103 °C -105 °C for 1 hour
- Store until needed
- Weigh immediately before use (Figure A)

(2) Sample analysis
- Shake the sample bottle vigorously at least 25 times (Figure B)
- Choose a sample volume that will yield a residue between 2.5 and 250 mg and pour the sample carefully into a graduate cylinder (Figure C)
- Filter the sample, and rinse the sides of the funnel at least twice with sterile buffered rinse water.
- Evaporate to dryness a drying oven (Figure D)
- When evaporating in a drying oven, lower temperature to approximately 2 °C below boiling to prevent splattering
- Dry evaporated sample for at least 1 hour in an oven at 103 °C to 105 °C
- Repeat cycle of drying and weighting until a constant weigh is obtained, or until weight change is less than 4 % of previous weight or 0.5 mg, whichever is less
- When weighing dried sample, be alert to change in weight due to air exposure and/or sample degradation
- Analyze at least 10 % of all samples in duplicate
- Duplicate determinations should agree within 5 % of their average weight

.4 Calculations

$$\text{mg total solids / L} = \frac{(A-B)\times 100}{\text{sample volume,mL}}$$

.5 Precision

Single-laboratory duplicate analyses of 41 samples of water and wastewater were made with a standard deviation of differences of 6.0 mg/L

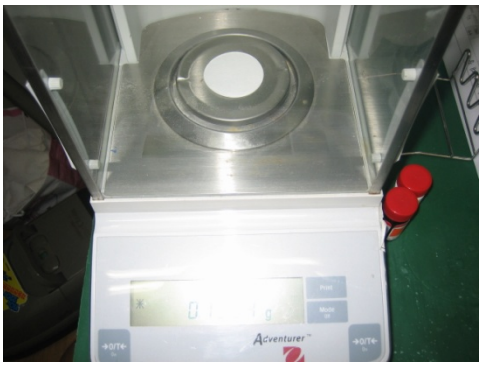


Figure A

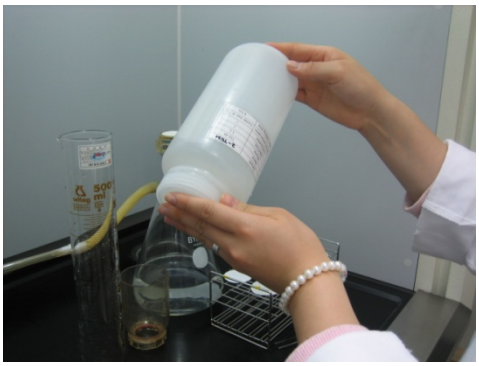


Figure B

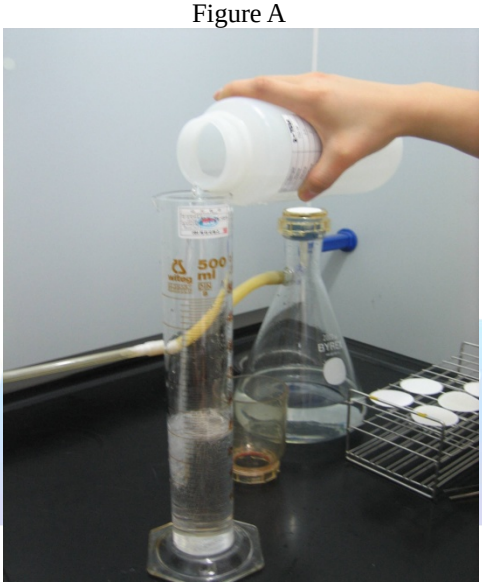


Figure C

Figure D

SOP: Total Coliform using membrane filter (APAH 9222B)

KOMERI SOP BWMS 20

Korea Marine Equipment Research Institute
Aquatic Organism Analytical Laboratory
1631-10 Songjeong-dong Gangseo-gu
Busan, Rep. of Korea

Prepared by Soo-Yeon Im
Approved by Young-Soo Kim
April 2011
Revision 4.0

.1. Scopes and Application

As related to the MF technique, the coliform group is defined as those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that develop red colonies with a metallic sheen within 24 h at 35 °C on an Endo-type medium containing lactose.

.2. Equipment and Supplies

- Glass lens with magnification of 2-5x, or stereoscopic microscope
- Hand tally or electronic counting device
- Pipettes, stainless steel, aluminum or borosilicate glass, for glass pipettes
- Ultraviolet unit for sanitization of the filter funnel between filtrations (optional)
- Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used
- Filter flask, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional
- Flask for safety trap placed between the filter flask and the vacuum source
- Forceps, straight or curved, with smooth tips to handle filters without damage
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles
- Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23
- Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids; or 15 x 60 mm, glass or plastic, with loose-fitting lids; or 15 x 100 mm
- Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10dilutions
- Flasks, borosilicate glass, screw-cap, 250-2000 mL volume
- Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size. Autoclave for 10 min at 121°C.
- Inoculation loops, at least 3 mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops. (Note: A platinum loop is required for the cytochrome oxidase test in the verification procedure.)
- Incubator maintained at 35 ± 0.5°C, with approximately 90% humidity if loose-lidded petri dishes are used
- Test tubes, 20 x 150 mm, borosilicate glass or plastic
- Test tubes, 10 x 75 mm, borosilicate glass
- Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes
- Test tubes screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size
- Whirl-Pak® bags

.3. Reagent and Standards

Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control.

Purity of Water: Reagent water conforming to Specification D1193, reagent water conforming Type II, Annual Book of ASTM Standards.

(1) *Phosphate Buffered Dilution Water*
(2) *LES Endo agar (Difco 0736 or 11203)*
(3) *M-Endo medium (Difco 0749 or 11119)*

.4. Procedure

(1) Prepare LES Endo Agar and M-Endo medium as directed in section 3.
(2) Mark the petri dish and report form with the sample identification and volume.
(3) Place a sterile membrane filter on the filter base, grid side up, and attach the funnel to the base so that the membrane filter is held between the funnel and the base (Figure A).
(4) Shake the sample bottle vigorously at least 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel (Figure B).
(5) Select sample volumes based on previous knowledge of the pollution level, to produce 20-80 colonies on the membranes. Sample volumes of 1-1,000 mL are normally tested. Smaller sample sizes or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions may be filtered, and the results may be combined.
(6) Filter the sample, and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum, and remove the funnel from the filter base (Figure C).
(7) Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the LES Endo Agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar (Figure D). Close the dish, invert, and incubate 35 ± 0.5°C for 22 to 24 h (Figure E).
(8) After 22-24 h, count and record the number of pink to dark-red color with a metallic surface sheen. Coliform (left) and non-Coliform (right) (Figure F).

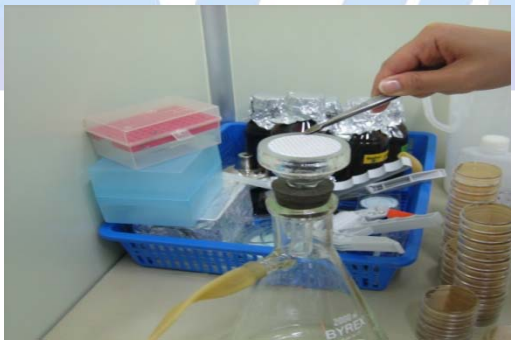


Figure A

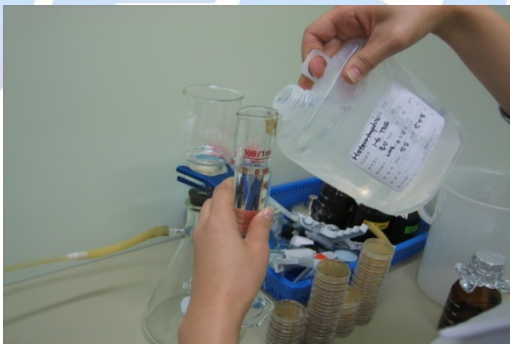


Figure B


Figure C


Figure D

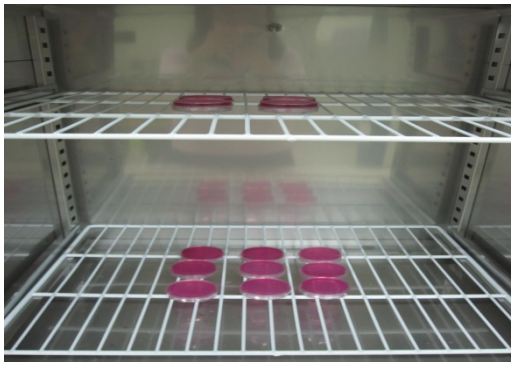


Figure E

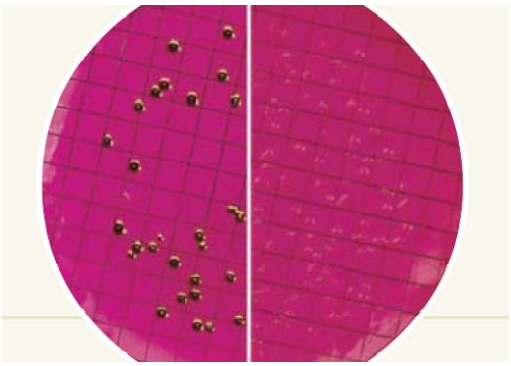


Figure F

.5. Data Analysis and Calculations

Use the following general rules to calculate the total coliform count per 100 mL of sample:

Select the membrane filter with an acceptable number of colonies (20-80), and calculate the number of total coliform per 100 mL according to the following general formula:

$$\text{total coliform / 100 mL} = \frac{\text{Number of total coliform colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

.6. Verification Procedure

Occasionally, typical sheen colonies may be produced by noncoliform organisms and atypical colonies (dark red or nucleated colonies without sheen) may be coliforms. Preferably verify all typical and atypical colony types. For drinking water, verify all suspect colonies by swabbing the entire membrane or pick a least five typical colonies and five atypical colonies from a given membrane filter culture. For waters other than drinking water, at a minimum, verify at least 10 sheen colonies (and representative atypical colonies of different morphological types) from a positive water sample monthly.

(1) Lactose fermentation – Transfer growth from each colony or swab the entire membrane with a sterile cotton swab (for presence-absence results in drinking water samples) and place in lauryl tryptose broth; Incubate the lauryl tryptose broth at 35±0.5 °C for 48 h. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose broth within 48 h verifies the colony as a coliform. Simultaneous inoculation of both media for gas production is acceptable. Inclusion of EC broth inoculation for 44.5±0.2 °C incubation will provide information on the presence of fecal coliforms. Use of EC-MUG with incubation at 44.5±0.2 °C for 24 h will provide information on presence of *E. coli*.

(2) Alternative coliform verifications – Apply this alternative coliform verification procedure to isolated colonies on the membrane filter culture. If a mixed culture is suspected or if colony separation If less than 2 mm, streak the growth to M-Endo medium or MacConkey agar to assure culture purity or submit the mixed growth to the fermentation tube method.

a) Rapid test – A rapid verification of colonies utilizes test reactions for cytochrome oxidase (CO) and β-galactosidase. Coliform reactions are CO negative and β-galactosidase positive within 4 h incubation of tube culture or micro (spot) test procedure.
b) Commercial multi-test systems – Verify the colony by streaking it for purification, selecting a well-isolated colony, and inoculation into a multi-test identification system for Enterobacteriaceae that includes lactose fermentation and/or β-galactosidase and CO test reactions.

Agenda	
Date/Time	
Place	

Participate Organization	Participant	Remarks
KOMERI		
KOMERI		
KOMERI		

QA person signature	Date/Time
Written by :	
Approved by :	
Dispatched by :	

Check List for Field Sampling (Ballasting) Project ID :

No.	Division	Analysis Item / Unit	Quantity	Check
1	Net (50 μm mesh)	≥ 50 μm	2	
2	Net (10 μm mesh)	≥ 10 to 50 μm	1	
3	Immersion Tank (1 m^3)	≥ 50 μm (netting)	1	√
4	Sieve (10 and 20 μm mesh)	≥ 10 to 50 μm	2	
5	Sieve (30 μm mesh)	≥ 10 to 50 μm	2	
6	Bottle (1 L-HDPE)	≥ 50 μm	7 ea. (day 0), 1 IMO, 3 treat, 3 con.	
7	Bottle (1 L-HDPE)	≥ 10 to 50 μm	7 ea. (day 0), 1 IMO, 3 treat, 3 con	
8	Bottle (1 L-PE)	DOC/POC	7 ea. (day 0), 1 IMO, 3 treat, 3 con	
9	Bottle (1 L-HDPE)	TSS	7 ea. (day 0), 1 IMO, 3 treat, 3 con	
10	Bottle (1 L-HDPE)	Extra	10	
11	Sterilized Pack (1 L-PE)	Bacteria group	7 ea. (day 0), 1 IMO, 3 treat, 3 con	
12	Sterilized Pack (1, 2 L-PE)	Extra	1 L-10 ea., 2 L-10 ea.	
13	COC Labeling	Each sample bottle	-	√
14	Turbidity meter	Turbidity	1	
15	Multi-probe (HydroLab)	pH, DO, Temp, Salinity, ORP, Conduct, Turbidity	1	
16	Thermometer	Extra	1	
17	Hand-held Refractometer	Extra	1	
18	pH/DO meter	Extra	1	
19	Beaker	5 L-HDPE	3	
20	Beaker	20 L-HDPE	3	
21	Graduate Cylinder	1 L-HDPE	2	
22	Microscope	Stereo	1	
23	Microscope	Converted	1	
24	Bogorov chamber	90 x 100 = 22 mL	1	
25	Bogorov chamber	110 x 90 = 36 mL	1	
26	Sedgewick-rafter chamber		1	
27	Hemacytometer		1	
28	Counter	Multi/single	3	
29	Pipette	1000 μL	2	
30	Sterilized pipette tip	1000 μL (Box)	2	
31	Pipette	10 mL	1	
32	Sterilized pipette tip	10 mL (Pack)	1	
33	Cornical Tube	50 mL	10	
34	1% Formalin	50 mL	1	
35	4% Formalin	50 mL	1	
36	Neutral Red	150 mL	1	
37	Squeeze Bottle		1	
38	Cooler box (-10 °C to 40 °C)	25 L (230 V and/or 12 V)	2	
39	Ice box (including ice pack)	40 L	2	
40	Heterotrophic Bacteria	20 L	2	
41	Box	48 L	3	
42	Seal tape (parafilm)	Roll	1	
43	Oil pen		2	
44	Scissor		1	
45	Lab tissue / towel		1	
46	Latex Glove		1	
47	Distilled water	10 L	1	
48	COC Binder		1	
49	H_2SO_4/Spuit		1	

	Name	Signature	Date/Time
Checked by :			
Approved by :			

Check List for Field Sampling (de-Ballasting)

Project ID :

No.	Division	Analysis Item / Unit	Quantity	Check
1	Net (50 μm mesh)	≥ 50 μm	2	
2	Net (10 μm mesh)	≥ 10 to 50 μm	1	
3	Immersion Tank (1 m^3)	≥ 50 μm (netting)	1	√
4	Sieve (10 and 20 μm mesh)	≥ 10 to 50 μm	2	
5	Sieve (50 μm mesh)	≥ 10 to 50 μm	2	
6	Bottle (1 L-HDPE)	≥ 50 μm	6 ea. (day 5), 3 treated, 3 control	
7	Bottle (1 L-HDPE)	≥10 to 50 μm	6 ea. (day 5), 3 control	
8	Bottle (10 L-HDPE)	≥ 10 to 50 μm	6 ea. (day 5), 3 treated	
9	Bottle (1 L-PE)	DOC/POC	6 ea. (day 5), 3 treated, 3 control	
10	Bottle (1 L-HDPE)	TSS	6 ea. (day 5), 3 treated, 3 control	
11	Bottle (1 L-HDPE)	Extra	10	
12	Sterilized Pack (1 L-PE)	Bacteria group	3 ea. (day 5), 3 control	
13	Sterilized Pack (2 L-PE)	Bacteria group	3 ea. (day 5), 3 treated	
14	Sterilized Pack (1, 2 L-PE)	Extra	1 L-10 ea., 2 L-10 ea.	
15	COC Labeling	Each sample bottle	-	√
16	Turbidity meter	Turbidity	1	
17	Multi-probe (HydroLab)	pH, DO, Temp, Salinity, ORP, Conduct, Turbidity	1	
18	Thermometer	Extra	1	
19	Hand-held Refractometer	Extra	1	
20	pH/DO meter	Extra	1	
21	Beaker	5 L-HDPE	3	
22	Beaker	20 L-HDPE	3	
23	Graduate Cylinder	1 L-HDPE	2	
24	Microscope	Stereo	1	
25	Counting chamber (zooplankton)	40 x 70 = 9 mL	2	
26	Counter	Multi/single	3	
27	Pipette	1000 μL	2	
28	Sterilized pipette tip	1000 μL (Box)	2	
29	Pipette	10 mL	1	
30	Sterilized pipette tip	10 mL (Pack)	1	
31	Cornical Tube	50 mL	10	
32	1% Formalin	50 mL	1	
33	4% Formalin	50 mL	1	
34	Neutral Red	150 mL	1	
35	Squeeze Bottle		1	
36	Cooler box (-10 °C to 40 °C)	25 L (230 V and/or 12 V)	2	
37	Ice box (including ice pack)	40 L	2	
38	Box	48 L	3	
39	Seal tape (parafilm)	Roll	1	
40	Oil pen		2	
41	Scissor		1	
42	Lab tissue / towel		1	
43	Latex Glove		1	
44	Distilled water	10 L	1	
45	COC Binder		1	
46	H_2SO_4/Spuit		1	

	Name	Signature	Date/Time
Checked by :			
Approved by :			

Sample Id.	
Name	TSS-B
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	TSS-M
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	TSS-E
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	DOC/POC-B
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	H_2SO_4
Anal. holding time	6 hrs

Sample Id.	
Name	DOC/POC-M
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	H_2SO_4
Anal. holding time	6 hrs

Sample Id.	
Name	DOC/POC-E
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	H_2SO_4
Anal. holding time	6 hrs

Sample Id.	
Name	Org. 10 to 50-B
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Org. 10 to 50-M
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Org. 10 to 50-E
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Org. 50-B
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Org. 50-M
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Org. 50-E
Volume	1 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	*V. cholerae*-B
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	In situ temp.
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	*V. cholerae*-M
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	In situ temp.
Pretreatment	None
Anal. holding time	6 hrs

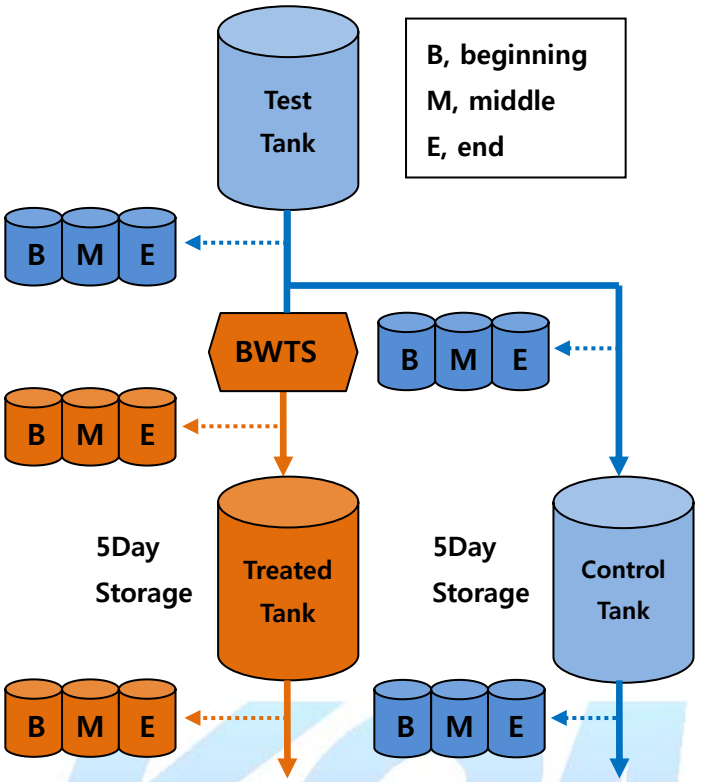
Sample Id.	
Name	*V. cholerae*-E
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	In situ temp.
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	*E.coli*-B
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	*E.coli*-M
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	*E.coli*-E
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

BWMS Test Procedure on Land-Based Test



<Standard for valid test>

Salinity in the test water	> 32 PSU	3-32 PSU	< 3 PSU
DOC	> 1 mg/L	> 5 mg/L	> 5 mg/L
POC	> 1 mg/L	> 5 mg/L	> 5 mg/L
TSS	> 1 mg/L	> 50 mg/L	> 50 mg/L

Density of organisms in the test water	
≥ 50 μm organisms	≥ 3 Phyla/Division 5 species ≥ 100,000 inds./m^3
≥ 10-50 μm organisms	≥ 3 Phyla/Division 5 species ≥ 1,000 inds./mL
Heterotrophic bacteria	≥ 10,000 cells/mL

Density of organisms in the treated water		
≥ 50 μm organisms		< 10 inds./m^3
≥ 10-50 μm organisms		< 10 inds./mL
Bacteria	Toxicogenic *Vibrio cholerae* (O1, O139)	< 1 cfu/100 mL
	Escherichia coil	< 250 cfu/100mL
	Intestinal *Enterococci*	< 100 cfu/100mL

Sample volume			
Ballasting	Test water	≥ 50 μm organisms	20 L × 3
		≥ 10-50 μm organisms	1 L × 3
		Bacteria	500 mL × 3
	Control water	≥ 50 μm organisms	20 L × 3
		≥ 10-50 μm organisms	1 L × 3
		Bacteria	500 mL × 3
	Treated water	≥ 50 μm organisms	1 m^3 × 3
		≥ 10-50 μm organisms	10 L × 3
		Bacteria	500 mL × 3
de-Ballasting	Control water	≥ 50 μm organisms	20 L × 3
		≥ 10-50 μm organisms	1 L × 3
		Bacteria	500 mL × 3
	Treated water	≥ 50 μm organisms	1 m^3 × 3
		≥ 10-50 μm organisms	10 L × 3
		Bacteria	500 mL × 3

Escherichia coil
Intestinal *Enterococci*
Total Coliform
→ Membrane filteration

Toxicogenic *V. cholerae* (O1, O139)
Heterotrophic bacteria
→ Spread-plate method

≥ 50 μm organisms → Stereomicroscopic investigation → Neutral red stain / Touch

≥ 10-50 μm organisms → 5-CFDA AM stain → Light microscopic investigation

Sample Id.	
Name	Enterococcus-B
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Enterococcus-M
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Enterococcus-E
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Heterotrophic-B
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Heterotrophic-M
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Id.	
Name	Heterotrophic-E
Volume	0.5 L
Date/Time	/
Site/Location	/
Sample preservation	Cooler box (4 °C)
Pretreatment	None
Anal. holding time	6 hrs

Sample Log Sheet

Project ID		**Test date**	
Kind of test	☐ Laboratory ☐ Pilot ☐ Land-based ☐ Shipboard		
Test salinity	☐ > 32 psu ☐ 3-32 psu ☐ < 3 psu		
Operation mode	☐ Ballasting ☐ de-Ballasting	**Test cycle**	
Test site		**Sample tracking**	Yes ( ) No ( )
Sampling manager		**Approved by**	

Division	Sample			Test water		Control water						Treated water								Personnel
	Bottle	Volume (L)				B		M		E		B		M		E				
Basic parameters (pH, DO, Salinity, Water temp., ORP, Turbidity)	HDPE beaker	20																		
TSS	HDPE bottle	1																		
DOC/POC	HDPE bottle	1																		
Organism (≥ 50 μm)	HDPE bottle after sieving with 50 μm plankton net	20 L	100 L																	
		20 L	1 m^3																	
Organism (≥ 10~50 μm)	HDPE bottle	1 L	10 L																	
Bacteria group (Heterotrophic bacteria, Coliform / *E. coli*, Intestinal Enterococci, Toxic *V. cholerae*)	Sterilized pack	1 L	2 L																	

* B, M and E indicate beginning, middle and end, respectively.

Tracking time (hh:mm)		
Collecting	Shipping	Arriving

Transit condition	
Shipped with	
Storage temp. (°C)	

COC Record / Field Sampling and Sample Transit Information

Project ID :		**Test site :**	
Date (YY/MM/DD) :		**Approved by :**	

WATER QUALITY SAMPLE

Sample ID	Temp (°C)	pH	ORP (mV)	Salinity (psu)	DO (mg/L)	Turbidity (NTU)	Time		Collect/Recorded by
							Collect	Record	
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									

Remarks :

Sample Receipt Form

Project Code : _____
Project No : _____

Approved: ☐ Authorized: ☐

Test (cycle/period) Number: _____ Client: _____
Received by: _____ Date/Time Received: _____
Number of Shipping Containers: _____

Shipment

Methods of delivery: _____ Tracking Numbers: _____
Chain of Custody (COC) Form: ☐ Shipped with samples ☐ No Forms

Cooler/Box

Container	Type	Sealed with	Seal condition	Container condition	Water temp. °C	Total samples

Samples

Labels:
☐ Sample labels agree with Chain of Custody forms
☐ Discrepancies (see Sample Custody Corrective Action Form)

Container seals:
☐ Tape ☐ Custody Seals ☐ Para films ☐ Other Seals (see Sample log)
☐ Seals intact for each shipping container
☐ Seals broken (see Sample log for impacted sample)

Sample Container:
☐ (HD)PE ☐ Glass Bottle ☐ PE-pack with Sterilization ☐ or Not ☐

Condition of sample
☐ Sample containers intact
☐ Sample containers broken/leaking (see Custody Corrective Action Form)

Sample temperature:
☐ Field condition _____°C ☐ Shipboard condition _____°C
☐ Receipt condition _____°C ☐ Lab (storage) condition _____°C

Temperature blank used: ☐ Yes ☐ No
(*Note: If temperature upon receipt differs from required conditions, see sample log comment field*)

Sample concentration: ☐ Yes ☐ No ☐ Unknown
Collection flow rate (or speed): _____ L/Min. ☐ Unknown
Sample volume: ☐ 500mL ☐ 1 L ☐ 2 L ☐ 10 L ☐ 20 L ☐ 1 ton ☐ Other
Sample collection method: ☐ Plankton net ☐ Sieving ☐ Sedimentation ☐ Other
Collected sample volume: ☐ 500mL ☐ 1 L ☐ 2 L ☐ 10 L ☐ 20 L ☐ 1 ton ☐ Other
Sample concentrated by: ☐ Plankton net ☐ Plankton Sieve ☐ Sedimentation ☐ Other
Analysis sample volume ☐ 3 mL ☐ 6 mL ☐ 12 mL ☐ 30 mL ☐ 60 mL ☐ Other

Sample Fixation: ☐ Yes ☐ No ☐ Unknown (with _____)
Sample pH: ☐ < pH 2 ☐ pH 3 ☐ pH 4 ☐ pH 5 ☐ pH 6 ☐ pH 7 ☐ pH 8 Other _____

Samples returned: ☐ Yes ☐ Discard ☐ Unknown/Lot No.: _____

Storage location: _____ **Storage time**: _____
Samples logged in by: _____ **Date/Time**: _____
Approved by: _____ **Approved on**: _____
Authorized by: _____ **Authorized on**: _____

Analysis Data Log Sheet

Project ID		**Test date**	
Kind of test	□ Pilot □ Land-based □ Shipboard		
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu		
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**	
Analyzed by		**Approved by**	

	Day 0 (YY. MM. DD)							Day 1 (YY. MM. DD)		Day 5 (YY. MM. DD)					
	Test water	Control			Treated			Control	Treated	Control			Treated		
		B	M	E	B	M	E			B	M	E	B	M	E
Water temp. (°C)															
pH															
ORP (mV)															
Salinity (psu)															
DO (mg/L)															
Turbidity (NTU)															
TSS (mg/L)															
DOC (mg/L)															
POC (mg/L)															
Organism (≥ 50 μm, inds./m^3)															
Organism (≥10-50 μm, inds./mL)															
Heterotrophic bacteria (cells/mL)															
Coliform (cfu/100 mL)															
E. coli (cfu/100 mL)															
Enterococci (cfu/100 mL)															
V. cholerae (cfu/100 mL)															
Remarks :															

*All data is arithmetic mean.

Test Cycle No:

KOMERI Project No:

Report Corrective Actions	**Corrective Action No:**
	Approved: **Authorized:**

COC Client:

COC Project:

COC Date:

Description of Problem		**Explanation**	
Custody		1.	
		2.	
		3.	

Document and Project Manager Notification			
Sample Custodian:		Date:	
Laboratory Manager:		Date:	
Project Manager		Date:	

Documentation of Client Notification

On _____ I phone called (meet at _____)

Results of Communication with Client (Describe any Corrective Action Directed by the Client)

Date this form was received back to the custodian: _____

Reference Number: _____

Heterotrophic bacteria

				Number of page :	
Project ID					
Test date		**Test time**		~	
Kind of test	□ Pilot □ Land-based □ Shipboard			**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu				% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**			
Analyzed by		**Approved by**			

Sample ID	Analyzed volume (mL)	Dilution factor (1×10^x)				Number of CFU on culture medium	Density (cell/mL)
		0	1	2	3		
						1	
						2	
						3	
						4	
						1	
						2	
						3	
						1	
						2	
						3	
						1	
						2	
						3	
						1	
						2	
						3	
						1	
						2	
						3	
						1	
						2	
						3	
						1	
						2	
						3	

Remarks
a. Incubate at 20 °C for 7 days, if NWRI is used.
b. Heterotrophic bacteria should be present in a density of at least10^4 living cell/mL in the test water.

Coliform / *Escherichia coli*

			Number of page :	
Project ID				
Test date		**Test time**	~	
Kind of test	□ Pilot □ Land-based □ Shipboard		**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu			% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**		
Analyzed by		**Approved by**		

Sample ID	Analyzed volume (mL)	Dilution factor (1×10^x)			Number of CFU on culture medium		Density (CFU/100 mL)	
		0	1	2	Coliform	*E. coli*	Coliform	*E. coli*
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		
					1	1		
					2	2		
					3	3		

Remarks
a. Coliform : Incubate at (35 ± 0.5) °C for 22 to 24 hours
E. coli : Incubate at (44.5 ± 0.2) °C for 20 to 24 hours
b. The average density of *E. coli* in the treated water is less than 250 CFU/100 mL.

Intestinal *Enterococci*

		Number of page :	
Project ID			
Test date		**Test time**	~
Kind of test	□ Pilot □ Land-based □ Shipboard	**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu		% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**	
Analyzed by		**Approved by**	

Sample ID	Analyzed volume (mL)	Dilution factor (1×10^x)			Number of CFU on culture medium	Density (CFU/100 mL)
		0	1	2		
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	

Remarks
a. Incubate at (41 ± 0.5) °C for 22 to 26 hours.
b. The average density of intestinal *Enterococci* in the discharge treated water is less than 100 CFU/100 mL.

Toxicogenic *Vibrio cholerae* (O1, O139)

		Number of page :	
Project ID			
Test date		**Test time**	~
Kind of test	□ Pilot □ Land-based □ Shipboard	**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu		% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**	
Analyzed by		**Approved by**	

Sample ID	Analyzed volume (mL)	Dilution factor (1×10^x)			Number of CFU on TCBS	Density (CFU/100 mL)
		0	1	2		
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	
					1	
					2	
					3	

Remarks
a. Incubate at 35 °C for 18 to 24 hours.
b. The average density of *Vibrio cholerae* in the discharge treated water is less than 1 CFU/100 mL or less than 1 CFU/1 g (wet weight) zooplankton samples.

Toxicogenic *Vibrio cholerae* (O1, O139)

				Number of page :	
Project ID					
Test date		**Test time**		~	
Kind of test	□ Pilot □ Land-based □ Shipboard			**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu				% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**			
Analyzed by		**Approved by**			

Sample ID	NB with 0% NaCl (+, -)	Poly antiserum	Number of colonies by IMViC
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		
	1		
	2		
	3		

Remarks
a. The average density of *Vibrio cholerae* in the discharge treated water is less than 1 CFU/100 mL or less than 1 CFU/1 g (wet weight) zooplankton samples.

Total suspended solid

		Number of page :	
Project ID			
Test date		**Test time**	~
Kind of test	□ Pilot □ Land-based □ Shipboard	**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu		% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**	
Analyzed by		**Approved by**	

Sample ID	Analyzed vol. (mL) (A)	Sample weight (mg)			*Total suspended solid (mg/L)	Average (mg/L)
		Before filtration (B)	After drying (C)	Value (C-B)		

Remarks : Test water standard is 5 mg/L in > 32 PSU and 50 mg/L in 3~32 PSU.

Organisms (≥ 10 – 50 µm)

			Number of page :		
Project ID					
Test date		**Test time**	~		
Kind of test	□ Pilot □ Land-based □ Shipboard			**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu				% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**			
Analyzed by		**Approved by**			

Sample ID	Concentrated volume (mL)		Number of Viable cells (cells/mL)	Average (cells/mL)
	Before	After		
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	
			1	
			2	
			3	

Treated water: (Viable cells × Concentration volume) / Sample volume

Remarks:

Organisms (≥ 10 – 50 µm)

Number of page :

Test (challenge) water		
Phyla/Division	Order	Genus/Species

Control water		
Phyla/Division	Order	Genus/Species

Treated water		
Phyla/Division	Order	Genus/Species

Organisms (≥ 10 – 50 μm)
(Re-growth analysis)

				Number of page :	
Project ID					
Test date		**Test time**	~		
Kind of test	□ Pilot □ Land-based □ Shipboard			**Test Environment**	°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu				% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**			
Analyzed by		**Approved by**			

Plate ID	Culture media	Culture condition		
		Temperature (°C)	Irradiance (μmol $m^{-2}s^{-1}$)	L : D Cycle

On Culture Plates (96 holes)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Organisms (≥ 50 μm)

				Number of page :	
Project ID					
Test date		**Test time**		~	
Kind of test	□ Pilot □ Land-based □ Shipboard		**Test**		°C
Test salinity	□ > 32 psu □ 3-32 psu □ < 3 psu		**Environment**		% R.H.
Operation mode	□ Ballasting □ de-Ballasting	**Test cycle**			
Analyzed by		**Approved by**			

Sample ID	Sample volume (L)	Concentrated volume (mL)	Analyzed volume (mL)	Viable Organism (individuals)	Average viable Organism (individuals/m^3)
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		
			1		
			2		
			3		

Organisms (≥ 50 μm)

Number of page :

Test water		
Phyla/Division	Order	Genus/Species
Diversity		Phyla/Division
		Species

Control water		
Phyla/Division	Order	Genus/Species

Treated water		
Phyla/Division	Order	Genus/Species

Total Residual Oxidant (TRO) concentration

Project ID					
Test date			**Test cycle**		
Kind of test	□ Pilot	□ Land-based	□ Shipboard	**Test Environment**	°C
Test salinity	□ > 32 psu	□ 3-32 psu	□ < 3 psu		% R.H.
Recorded by			**Approved by**		

Operation mode	Ballasting					
Time (hh:mm)	TRO (mg/L as Cl_2)	Voltage (V)	Current (A)	Conductivity (mS/cm)	Flow rate (m^3/hr)	Remark

Plate ID	Culture media	Culture condition		
		Temperature (°C)	Irradiance (μmol $m^{-2}s^{-1}$)	L : D Cycle

	Incubation time (day)										
Hole No.	1	2	3	4	5	6	7	8	9	10	Remarks

○ : Re-growth
× : Not Re-growth

Operation mode	De-Ballasting			
Time (hh:mm)	TRO (mg/L as Cl_2)	Conductivity (mS/cm)	Flow rate (m^3/hr)	Remark

Standard Operation Procedures (SOP)

for

Chemical Analysis

Appendix 3.4.2

1. Total Organic Carbon - DOC, POC : ISO 8245:1999

1). Scope and Application

This test method is a way to measure total organic carbon(TOC) and dissolved organic carbon(DOC) in drinking water, ground water, surface water, sea water and waste water. The organic carbon in sample flows injection line, combustion tube, detector. The method applies to water samples containing organic carbon content ranging from 0.3 mg/L to 1 000 mg/L. This is the measurement method with Total organic carbon analyzer using Non-dispersive infrared absorption detector.

2). Equipment and Apparatus

- ***For analysis equipment***: autosampler(ASI-V, shimadz), total organic carbon analyzer(TOC-V_{CPH}) (Figure A). Combustion tube with catalyst is oxidized to carbon dioxide by combustion. Non-dispersive infrared absorption detector (NDIR) was used for quantitative analysis of organic carbon. The details of analysis condition were described in Table 1

Figure A. Autosampler and Total organic carbon analyzer

Table 1. Total drganic carbon analyzer condition for DOC/POC

Variable	Valve
Combustion Temp.	680 °C

3). Reagent and Standards

- ***Standard solution (1 000 mg/L) :*** Dissolve 2.125 g of potassium hydrogen phthalate($C_8H_5KO_4$) (dried for 1 h at a temperature between 105° C and 120 °C) in a 1 000 mL one-mark volumetric flask filled with 700 mL of de-ionized water, then dilute to volume with de-ionized water. The solution is stable for about 2 months if stored in a tightly stoppered bottle in a refrigerator.
- ***2N HCl***
- ***H_2SO_4***

4). Sampling and preparation of the water sample

- ***Sampling:*** When sampling, ensure that the samples being collected are representative (particularly in the presence of undissolved substances), and take care not to contaminate the

samples with organic substances. Collect water samples in glass or polyethylene bottles, completely filled with the sample, and, if biological activity is suspected, acidify to pH 2. If volatile organic compounds are suspected, carry out the measurement without acidification and within 8 h of sampling. Otherwise, store the sample in a refrigerator at a temperature in the range of 2 °C to 5 °C, and analyse within 7 days. If this is not possible, the sample can be kept at –15 °C to –20 °C for several weeks.

- ***Preparation of the water sample:*** If the sample is not homogeneous and it is not possible to obtain a representative sample, even after thorough shaking, use an appropriate apparatus to homogenize the sample. If only dissolved organic substances (DOC) are to be determined, filter the sample through a membrane filter, 0.45 µm pore size, which has previously been washed with hot water to completely remove adhering organic substances.

5). Procedure

- ***Calibration:*** When making Calibration curve, the minimum quantitation range becomes 1 ~ 20 mg L^{-1} to verify a linearity of Calibration curve. However, in case measured value exceeds this concentration range, sample is diluted and then analyzed once more.

a) Pipette 100 mL of the potassium hydrogen phthalate stock solution into a 1 000 mL one-mark volumetric flask, and dilute to volume with de-ionized water (= 100 mg/L).
b) After that, standard solution (100 mg/L) pipette into each of a series of 100 mL volumetric flasks for example 0 mL, 1 mL, 2 mL, 5 mL, 10 mL, 20 mL of potassium phthalate solution (100 mg/L), and dilute to volume with de-ionized water. The concentration of standard solution is applicable to 1, 2, 5, 10, 20 mg L-1. The amount and number of standard solution to be used can be different as necessary.
c) As a analysis result, the sample should be diluted and analyzed again if this Calibration curve gets out of concentration range.

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard
- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

****(law data : KTR–Val–DOC/POC)***

2. Bromide Chlorate and Chloride : US EPA 300.1 : 1997

1). Scope and Application

This method covers the determination of the following inorganic anions in reagent water, surface water, ground water, and finished drinking water. As a result of different specified injection volumes (See conditions in Tables 1A and 1B), these anions are divided between the common anions. These different injection volumes are required in order to compensate for the relative concentrations of these anions in drinking water and maintain good chromatographic peak shape throughout the expected dynamic range of the detector.

2). Equipment and Apparatus

- ***Ion chromatograph*** -- Analytical system complete with ion chromatograph and all required accessories including syringes, analytical columns, compressed gasses and a conductivity detector.
Anion guard column: Dionex AG18-HC, 2 mm (P/N 52248), or equivalent. This column functions as a protector of the separator column. If omitted from the system the retention times will be shorter.
Anion separator column: Dionex AS18-HC column, 2 mm (P/N 52244), or equivalent.
Anion suppressor device: (ASRS, P/N 43187). An equivalent suppressor device may be utilized by a combined baseline drift/noise of no more than 5 nS per minute over the background conductivity.
The ASRS was set to perform electrolytic suppression at acurrent setting of 100 mA using an external source DI water mode. Insufficient baseline stability was observed using the ASRS in recycle mode.
- ***Detector*** -- Conductivity cell (Dionex CD20, or equivalent).

Figure A. Ion chromatograph system

3). Reagent and Standards

- ***Reagent water***: Distilled or deionized water, free of the anions of interest. Water should contain particles no larger than 0.20 microns.
- ***Eluent solution***: KOH 23 Mm.
This eluent solution must be purged for 15 minutes with helium prior to use to remove dissolved gases which may form micro bubbles in the IC compromising system performance and adversely effecting the integrity of the data.
- ***Stock standard solutions, 1000mg/L (1 mg/mL):*** Stock standard solutions may be purchased as

certified solutions.
Bromide (Br^-) 1000 mg/L: Accu Standard.
Chlorate (ClO_3^-) 1000 mg/L: Dissolve 1.27 g of $NaClO_3$ and mess up 1 L with deionized water.

STOCK STANDARD SOLUTION (SSS)

Chemical Abstract Services	STD1	STD2	STD3	STD4
Br^-	1 mg/L	2 mg/L	5 mg/L	10 mg/L
ClO_3^-	1 mg/L	2 mg/L	5 mg/L	10 mg/L

4). Sampling and preparation of the water sample

- ***Sampling:*** Samples should be collected in plastic or glass bottles. All bottles must be thoroughly cleaned and rinsed with reagent water. Volume collected should be sufficient to insure a representative sample, allow for replicate analysis, if required, and minimize waste disposal.
- Special sampling requirements and precautions for chlorite.

Sample bottles used for chlorite analysis must be opaque to protect the sample from light.

5). Procedure

- ***Calibration:***. When making Calibration curve, the minimum quantitation range becomes 1 ~ 10 mg L^{-1} to verify a linearity of Calibration curve. However, in case measured value exceeds this concentration range, sample is diluted and then analyzed once more.

d) The concentration of standard solution is applicable to 0, 1, 2, 5, 10 mg L^{-1}. The mixed internal standard solution (1,000 mg L^{-1}) is added to sample by taking 5 mL correctly. The amount and number of standard solution to be used can be different as necessary.
e) As a analysis result, the sample should be diluted and analyzed again if this Calibration curve gets out of concentration range.

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard
- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

*****(law data : KTR–Val–Br^- & ClO_3^-)***

3. Bromate : ISO 15061 : 2001

1). Scope and Application

This method is a way to simultaneously measure bromate. Bromate is measured using a High Performance Liquid Chromatography with the addition of a postcolumn reagent. The method uses an Ionpac AS9-HC column and followed by postcolumn addition of *o*-dinisidine to visible absorbance detection of the bromated.

2). Equipment and Apparatus

- ***For analysis equipment***: High Performance Liquid Chromatography (HPLC, Agilent 1100 series) with the addition of a postcolumn reaction derivatization instrument (PCX 5200, Pickering). (Figure A). The details of analysis condition were described in Table 1.

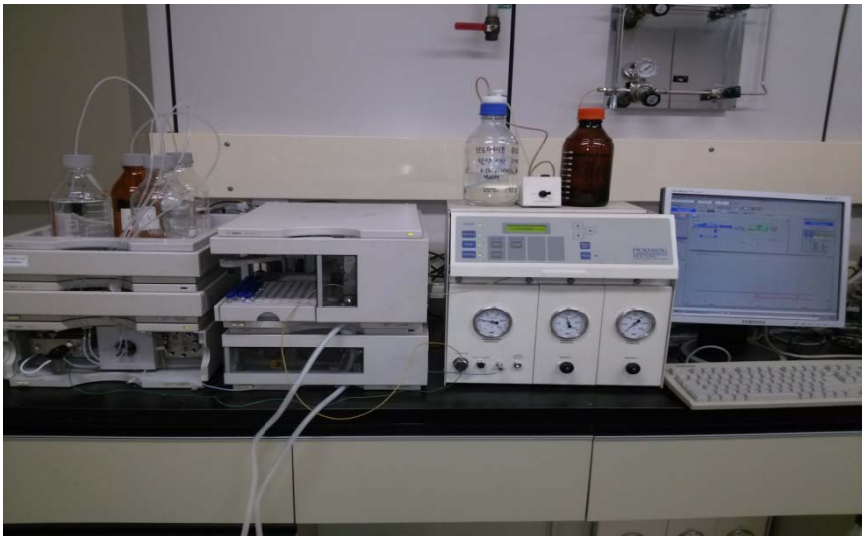


Figure A. Purge & Trap with Gas chromatograph Mass spectrometric detector

Table 1. GC/MSD condition for VOCs/THMs

Column	Dionex AG9-HC, 50 mm guard column, Dionex AC9-HC, 250 mm analytical column
Eluent solution	9 mM sodium carbonate
Flow rate	0.7 mL/min
Sample volume	500 uL
Detection	Absorbance at 450 nm
Postreactor temp.	60 ℃

3). Reagent and Standards

- ***Standard solution (1000 mg/L):*** Prepare stock solution by dissolving 0.1180 g Sodium bromate in reagent water and dilute to 100 mL.
- ***Postreagent*** : Add 40mL of 70% nitric acid to about 300 mL reagent water in a 500 mL volumetric flask. Add 2.5 g KBr and stir to dissolve. Dissolve 250 mg of *o*-Dianisidine dihydrochloride salt in 100 mL methanol and add to the nitric acid/KBr solution. Bring to volume with reagent water. Prepare in advance, set aside overnight until the slight champagne color fades, and filter through a 0.45 ㎛ filter.

- ***Ethylenediamine*** : Preservation Solution : Dilute 2.8 mL of ethylenediamine(99%) to 25 mL with reagent water.

4). Sampling and preparation of the water sample

- ***Sampling:*** Add 5 mg Ethylenediamine. If analysis cannot be performed within 2 hr from time of sampling, samples should be kept cool (4℃)

5). Procedure

- ***Calibration:*** When making Calibration curve, the minimum quantitation range becomes 5 ~ 500 μg L^{-1} to verify a linearity of Calibration curve. However, in case measured value exceeds this concentration range, sample is diluted and then analyzed once more.

f) Homogenize the sample in a blender.
g) 2.0 mL of sample elute cartridges step by step (Ba-cartridge, Ag-cartridge)
h) Sample solution filtered through a 0.45 um filter.
i) Analysis according to chromatography condition.(table 1.)

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard
- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

****(law data : KTR–Val–Bromate)***

4. Perchlorate: US EPA 331.0 : 2005

1). Scope and Application

This test method is a way to simultaneously measure perchlorate among active materials created in BWMS. Perchlorate in sample is determined by a liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). This test method can be used to acquire data using multiple reaction monitoring (MRM) detection.

2). Equipment and Apparatus

- ***For analysis equipment***: LC/ESI-MS/MS, 1200 series HPLC with 6410 series triple quadruple mass spectrometer (QqQ) (Agilent Technologies, CA, USA) (Figure A). The details of analysis condition were described in Table 1. MRM was used for quantitative analysis and the ions to be selected were shown in Table 2.

Figure A. Liquid chromatograph Mass spectrometric detector

Table 1. LC/ESI-MS/MS condition for perchlorate

Variable	Value
COLUMN	IonPac® AS 21 (2 x 250 mm; DIONEX)
Mobile phase	200 mM Methylamin in Water
Flow rate	0.3 mL/min
Injection Vol.	50 µL
Run time	7 min
Ionization mode	Negative Ion Electrospray
Gas	Nitrogen, 350 °C, 11 L/min
Capillary Volt.	3500 V
Nebulizer Press.	40 psig

Table 2. Retention time, selected ions and MRM condition

Compound	Retention time (min)	Precusor ion (m/z)	Product ion (m/z)	Fragment energy (V)	Collision energy (eV)
Perchlorate	4.73	99, 101	83, 85	110	24
Perchlrate-$^{18}O_4$	4.73	107, 109	89, 91	110	24

3). Reagent and Standards

- ***Perchlorate standard solution :*** ClO_4^- (AccuStandard Cat. No. IC-PER-10X-1)
- ***Internal standard solution :*** $Cl^{18}O_4^-$ (CIL Cat. No. OLM-7310-1.2)
- ***De-ionized water*** : using Milli-Q system
- ***Methylaminew :*** 40 wt.% in water (Aldrich Cat. No. 426466 or equivalent)

4). Sampling and preparation of the water sample

- ***Sampling:*** Samples must be collected in accordance with conventional sampling practices. Samples should be confirmed to be at or below 10 °C. Samples stored in the lab must be held at or below 6 °C until analysis. Samples should not be frozen.
- ***Preparation of the water sample:*** For the water samples, treat with serially placed OnGuard II cartridges (barium and silver) to remove sulfate and chloride ions. Finally, samples are filtered through a 0.2-μm membrane filter. Each sample is spiked with 1.0 μg L^{-1} of internal standard.

5). Procedure

- ***Calibration:*** When making Calibration curve, the minimum quantitation range becomes 0.01 ~ 10 μg L^{-1} to verify a linearity of Calibration curve (for example, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg L^{-1}). Internal standard's concentration is 1.0 μg L^{-1}. However, in case measured value exceeds this concentration range, sample is diluted and then analyzed once more.

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard.
- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14.
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

****(law data : KTR–Val–PER)***

5. Volatitle organic compounds: US EPA 524.2 : 1994

1). Scope and Application

This test method is a way to simultaneously measure volatile organic compounds among active materials created in BWMS. The volatile organic compound in sample is purged by an inert gas, extracted, absorbed and concentrated into Trap, and then heated and desorbed. This is the measurement method with Gas chromatograph-Mass spectrometry using column.
This test method is applied to analyze total 30 compounds except dichloromethane, halogen volatile organic compound under water. The detailed items are shown in Table 2.

2). Equipment and Apparatus

- ***For analysis equipment***: a purge and trap device, pretreatment device of TEKMAR Corp., with a spectrometric detector (GC/MSD, Agilent 6890 GC and 5973 series MSD; Agilent Technologies, CA, USA) (Figure A). Electron impact (EI) was used for ionization method and ionization energy was 70 eV. Selected ion monitoring (SIM) was used for quantitative analysis and the ions to be selected were shown in Table 4. The details of analysis condition were described in Table 1,2

Figure A. Purge & Trap with Gas chromatograph Mass spectrometric detector

Table 1. Purge & Trap condition for VOCs/THMs

Variable	Value	Variable	Value
Valve oven Temp.	140 °C	Dry Purge Flow	100 mL/min
Transfer Line Temp.	140 °C	Dry Purge Temp.	20 °C
Sample Mount Temp.	90 °C	Desorb Preheat	250 °C
Purge Ready Temp.	35 °C	Desorb Temp.	250 °C
Condenser Ready Temp.	40 °C	Desorb Time	2.00 min
Condenser Purge Temp.	20 °C	Desorb Flow	400 mL/min
Purge Time	11 min	Trap Bake Temp.	290 °C
Purge Flow	40 mL/min	Trap Bake Time.	5.0 min
GC Start	Start of Desorb	Trap Bake Flow	400 mL/min
Dry Purge Time	1.00 min	Condenser Bake Temp.	225 °C

Table 2. GC/MSD condition for VOCs/THMs

Variable	Value
GC COLUMN/GAS	DB-VRX (J&W Scientific) , 60 m x 0.25 mm x 0.25 µm / He at 0.8mL/min(확인)
GC Injector	Split ratio: 10:1, Temp : 150 °C
GC Oven	Int. temp. 35 °C, hold 7.00 min ; Ramp rate of 5.00 °C /min to 100 °C, hold for 0.00 min; Ramp rate 15 °C/min to 150, hold for 3.00 min; (Total run time of 36.33 min)
Mass spec	Interface at 250 °C, MS quad 150 °C ; MS source at 230 °C, low mass 35 amu

3). Reagent and Standards

- ***Standard solution :*** 30 volatile organic compounds and 3 kinds of internal standards (deuterated standards and Fluorobenzene) were purchased from AccuStandard Inc., CT, USA. (Table 1), All organic solvents used for experiments including dilution and purification are HPLC grade solvents of J.T. Baker (Phillipsburg, NJ) and de-ionized water which has been run through Milli-Q system was used.
- ***HCl(1+1)***
- ***Sodium thiosulfate pentahydrate***

Table 3. Test item

Compounds	CAS Number	Compounds	CAS Number
1,1-Dichloroethene	75-35-4	Dibromochloromethane	124-48-1
Dichloromethane	75-09-2	Tetrachloroethene	127-18-4
trans-1,2-Dichloroethene	156-60-5	1,1,1,2-Tetrachloroethane	630-20-6
1,1-Dichloroethane	75-34-3	1,2,3-Trichloropropane	96-18-4
cis-1,2-Dichloroethene	156-59-2	Chlorobenzene	108-90-7
Bromochloromethane	74-97-5	Tribromomethane	75-25-2
Trichloromethane	67-66-3	1,1,2,2-Tetrachloroethane	75-34-5
1,2-Dichloroethane	107-06-2	Bromobenzene	108-86-1
1,1,1-Trichloroethane	71-55-6	2-Chlorotoluene	95-49-8
Tetrachloromethane	56-23-5	4-Chlorotoluene	106-43-4
Dibromomethane	74-95-3	1,2-Dibromo-3-chloropropane	96-12-8
1,2-Dichloropropane	95-49-8	1,2,4-Trichlorobenzene	120-82-1
Dichlorobromomethane	75-27-4	1,2,3-Trichlorobenzene	87-61-6
1,1,2-Trichloroethane	79-00-5	1,3,5-Tribromobenzene	626-39-1
Dibromochloromethane	124-48-1	1,2,4-Tribromobenzene	615-54-3

4). Sampling and preparation of the water sample

- ***Sampling:*** Sample was adjusted by adding sodium thiosulfate(25mg) and one drop(10mL) of sulfuric acid(1+5) to a glass bottle in order to remove residual chlorine. The sample was collected about 100mL without any space and airtightly sealed. All the samples were collected in the same manner.. Collect water samples in glass, completely filled with the sample, The sample collected at site was transported in the icebox to make it kept the sample in a refrigerator at a temperature in the range of 2 °C to 5 °C. All samples were analyzed within 7 days.

- ***Preparation of the water sample:*** If only dissolved organic substances (DOC) are to be determined, filter the sample through a membrane filter, 0,45µm pore size, which has previously been washed with hot water to completely remove adhering organic substances.

5). Procedure

- ***Calibration:*** When making Calibration curve, the minimum quantitation range becomes 1 ~ 500 μg L^{-1} to verify a linearity of Calibration curve. However, in case measured value exceeds this concentration range, sample is diluted and then analyzed once more.

  a5mL of purified water are accurately injected to Spadure by using automatic injector. (In some cases, the amount of sample may be increased.)

  a) After that, mixed standard solution (1.0 mg/L) of volatile organic compounds is inserted by degrees of 0,5, 10, 50, 100, 250, 500, 1000, 25000 μL. The mixed internal standard solution (1.0 mg/L) is added to sample by taking 50 μL correctly. The concentration of standard solution is applicable to 0,1, 2, 10, 20, 50, 100, 200, 500 μg L-1. The amount and number of standard solution to be used can be different as necessary.
  b) As a analysis result, the sample should be diluted and analyzed again if this Calibration curve gets out of concentration range.
  c) The volatile organic compound is purged at an uniform temperature, collected from Trap, heated and desorbed, and then injected with Gas chromatograph Mass spectrometric

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard
- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

(law data : KTR–Val–VOC)

6. Haloacetic acids: US EPA 522.2 : 1995

1). Scope and Application

This is a gas chromatographic (GC) method applicable to the determination of the listed halogenated acetic acids in drinking water, ground water, raw source water and water at any intermediate treatment stage. In addition, chlorinated herbicide, Dalapon, may be determined using this method. This test method is applied to analyze total 10 compounds under water. The detailed items are shown in Table 2.

2). Equipment and Apparatus

- ***For analysis equipment***: Gag chromatograph (Agilent 7890A GC System), with Autosampler (Agilent 7683B Injector). The details of analysis condition were described in Table 1

Figure A. Gas chromatograph

Table 1. GC condition for HAAs

Variable	Value
GC COLUMN/GAS	DB-5MS (J&W Scientific) , 30 m x 0.25 mm x 0.25 µm / He at 0.8 mL/min
GC Injector	Split ratio: 10:1, Temp : 210 °C
GC Oven	Int. temp. 40 °C, hold 5.00 min ; Ramp rate of 2.5 °C /min to 65 °C, hold for 0.00 min; Ramp rate 10 °C/min to 85, hold for 0.00 min; Ramp rate 20 °C/min to 205, hold for 7.00 min; (Total run time of 30.00 min)
Detector	Temp : 280 °C

3). Reagent and Standards

- ***Standard solution*** *:* Haloacetic acid standards, internal standard(1,2,3-Trichloropropane), surrogate standards (2,3-Dibromopropionic acid and 2-bromobutanoic acid) were purchased from

AccuStandard Inc., CT, USA. (Table 2), All organic solvents used for experiments including dilution and purification are HPLC grade solvents of J.T. Baker (Phillipsburg, NJ) and de-ionized water which has been run through Milli-Q system was used.

- ***Solent :*** Methanol, Acetone, MTBE
- ***Sulfuric acid ,*** H_2SO_4
- ***Sodium sulfate,*** Na_2SO_4
- ***Copper II sulfate pentahydrate,*** $CuSO_4 \cdot 5H_2O$
- ***Sodium bicarbonate,*** $NaHCO_3$
- ***Ammonium chloride,*** NH_4Cl

Table 2. Test item

Compounds	CAS Number
Monochloroacetic acid (MCAA)	79-11-8
Monobromoacetic acid (MBAA)	79-08-3
Dichloroacetic acid (DCAA)	79-43-6
Dalapon	75-99-0
Trichloroacetic acid (TCAA)	76-03-9
Bromochloroacetic acid (BCAA)	5589-96-8
Dibromoacetic acid (DBAA)	631-64-1
Bromodichloroacetic acid (BDCAA)	71133-14-7
Chlorodibromoacetic acid (CDBAA)	5278-95-5
Tribromoacetic acidd (TBAA)	75-96-7
1,2,3-Trichloropropane (IS)	96-18-4
2,3-Dibromopropionic acid (SS)	600-05-5
2-Bromobutanoic acid (SS)	80-58-0

4). Sampling and preparation of the water sample

- Sampling

a) Grab Samples were collected using amber glass container with TFE-lined screw-caps and capacities of 1 000 mL.
b) Prior to shipment to field, adds ammonium chloride (NH_4Cl) to the sample bottle in an amount to produce a concentration of 100 mg/L and hydrochloric acid (HCl) 4 drops.
c) Seal the bottle and agitate by hand for 1 min.
d) The collected sample at site was transported in the icebox to make it kept the sample in a refrigerator at a temperature in the range of 2 °C to 5 °C. All samples were analyzed within 7 days from extraction if stored at 4 °C or within 14 days if stored at -10 °C or less.

- Preparation of the water sample

a) Remove the samples from storage and allow them to equilibrate to room temperature.
b) Place 50 mL of water sample in 125 mL *separatory funnel. Adjust the pH to less than 0.5 by adding at least 2 mL of concentration sulfuric acid.*
c) *Cap shake and then check the pH with a pH meter or pH paper.*
d) *Quickly add approximately 2 g of* Copper II sulfate pentahydrate and shake until dissolved.
e) Quickly add 16 g of anhydrous sodium sulfate and shake for 3 to 5 minutes until almost all is dissolved.
f) Add 5 mL MTBE and vigorously shake for 2 minutes by hands. Allow the phases to separate for approximately 5 minutes.

- Methylation

a) Using a pasteur pipet, transfer approximately 4 mL of the upper MTBE layer to a 15 mL graduated glass vial.
b) Add 1 mL 10 % sulfuric acid in methanol to each vial.

c) Cap and place in heating water bath at 50 °C and maintain for 2 h. Remove the vial from the Heating water bath and allow them to cool before removing the caps.
d) Add 4 mL saturated sodium bicarbonate solution. Shake each vial for 2 minutes. As the neutralization reaction moves to completion, it is important to continue by venting frequently to release the evolved CO_2.
e) Transfer exactly 1.0 mL of the upper MTBE layer to an autosampler vial. Analyze the samples as soon as possible.

5). Procedure

- ***Calibration:*** When making Calibration curve, the minimum quantitation range becomes 20 ~ 2 000 μg L^{-1} to verify a linearity of Calibration curve. However, in case measured value exceeds this concentration range, sample is diluted and then analyzed once more.

d) Place 50 mL of purified water in 125 mL *separatory funnel.* Mixed standard solution (10 mg/L) of haloacetic acids is inserted by degrees of 10 ~ 1 000 μL. The surrogate standard solution (10 mg/L) is exactly spiked 500 μL. Each standard solution is extracted the same ways as sample preparation and methylation. The concentration of standard solution is applicable to 20 ~ 2 000 μg L^{-1}. The amount and number of standard solution to be used can be different as necessary.
e) As a analysis result, the sample should be diluted and analyzed again if this Calibration curve gets out of concentration range.
f) The internal standard solution (10 mg/L) is added 20 μL exactly. Sample of autosampler vial is injected with gas chromatograph.

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard

- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14

- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation

- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

(law data : KTR–Val–HAA_1-10)
KTR-Val-HAA_1 : MCAA
KTR-Val-HAA_2 : MBAA
KTR-Val-HAA_3 : DCAA
KTR-Val-HAA_4 : Dalapon
KTR-Val-HAA_5 : TCAA
KTR-Val-HAA_6 : BCAA
KTR-Val-HAA_7 : DBAA
KTR-Val-HAA_8 : BDCAA
KTR-Val-HAA_9 : CDBAA
KTR-Val-HAA_10 : TBAA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7. Haloacetonitriles : US EPA Method 551.1 : 1995

1). Scope and Application

This method is applicable to the determination of the following analytes infinished drinking water, drinking water during intermediate stages of treatment, and raw source water. The particular choice of analytes from this list should be a function of the specific project requirements.

2). Equipment and Apparatus

- ***For analysis equipment***: The final extracts were analyzer by GC(Agilent 6890 series) equipped with a linearized electron capture detector (ECD), fused silicacapillary column(Figure A). The details of analysis condition were described in table1.

Figure A. Gas chromatograph with electron-capture detection.

Table 1. GC/ECD condition for HANs.

Inlet Temp.	250 °C
GC COLUMN/GAS	DB-5MS (J&W Scientific) , 60 m x 0.25 mm x 0.25 μm / He at 0.7 mL/min
Injection	2 μL
Detector Temp.	280 °C
GC oven	Int. temp. 50 °C, hold for 0.00 min ; Ramp rate of 5.00 °C /min to 110 °C, hold for 0.00 min; Ramp rate 15 °C/min to 230 °C, hold for 10.00 min;

3). Reagent and Standards

- ***Standard solution :*** HANs(Table 1) and internal standards(p-Bromofluorobenzene), surrogate standard(decafluorobiphenyl) were purchased from AccuStandard Inc., CT, USA., All organic solvents used for experiments including dilution and purification are HPLC grade solvents of J.T. Baker(Phillipsburg, NJ) and de-ionized water which has been run through Milli-Q system was used.

- Methyl tert-butyl ether(MTBE Merck)
- Amonium chloride
- Sodium chloride
- Sodium sulfate

Table 3. Test item

Compounds	CAS Number
Trichloroacetonitrile	545-06-5
Dichloroacetonitrile	3018-12-0
Chloral Hydrate	75-87-6
Chloropicrin	76-06-2
Bromochloroacetonitrile	83463-62-1
Dibromoacetonitrile	3252-43-5

4). Sampling and preparation of the water sample

- ***Sampling:*** Add 10 mg ammonium chloride and 1-2 drop 6 N hydrochloric acid solution If analysis cannot be performed within 2 hr from time of sampling, sample should be kept cool (4°C). The samples are stable for 14 days.

5). Procedure

a) Homogenize the sample in a blender.
b) After 500 mL of sample add to 500mL separate funnel, 50 μL surrogate standard solution Spike.
c) The separate funnel is sealed and shaken for 4 min.
d) 10 g sodium chloride and 40 mL MTBE add to separate funnel.
e) After the extract solution evaforate until 0.5 mL, spike 100 μL internal standard solution and dilute to 1 mL.

6). Quality Control

- ***Calibration:*** Initial calibration should be conducted with at least five different concentrations of target analytical standard
- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves, and as a data from manufacturer. or Detection limit was calculated value that S × 3.14
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

***(law data : KTR–Val–HAN)**

8. Halophenols : US EPA Method 8041A :2007

1). Scope and Application

This method is a way to simultaneously measure **hallogenated** phenols. this method also includes a procedure for the derivatization of the phenols and identification of the target phenols aspentafluorobenzylbromoether derivatives (PFBBr). And phenols were analyzed by GC MS.

2). Equipment and Apparatus

- ***For analysis equipment***: The target phenols were analyzed by gas chromatograph mass spectrometer (shimazu, QC 2010plus)(Figure A). Selected ion monitoring (SIM) was used for quantitative analysis and the ions to be selected were shown in Table 3. The details of analysis condition were described in Table 1.

Figure A. Gas chromatograph Mass spectrometer (shimazu, QP 2010plus)

Table 1. GC/MSD condition for hallophenols

Variable	Valve
GC COLUMN/GAS	DB-5MS UI (J&W Scientific) , 60 m x 0.25 mm x 0.25 μm / He at 1.0 mL/min
GC Injector	Temp : 260 °C, 3 uL
GC Oven	Int. temp. 110 °C, hold 2.00 min ; Ramp rate of 15.00 °C /min to 250 °C, hold for 11.00 min; Ramp rate 30 °C/min to 340, (Total run time of 36.33 min)
Mass spec	Interface at 280 °C, MS source at 260 °C

3). Reagent and Standards

- ***Standard solution :*** 22 halogenated phenol compounds and internal standards (deuterated standards and 2,5-dibromotoluene) were purchased from AccuStandard Inc., CT, USA. (Table 2), All organic solvents used for experiments including dilution and purification are HPLC grade solvents of J.T. Baker (Phillipsburg, NJ) and de-ionized water which has been run through Milli-Q system was used.

Table 2. Target compounds.

Compounds	CAS Number
2-Chlorophenol	95-57-8
3-Chlorophenol	108-43-0
4-Chlorophenol	106-48-9
2,6-Dichlorophenol	87-65-0
2,5-Dichlorophenol	583-78-8
2,4-Dichlorophenol	120-83-2
3,5-Dichlorophenol	591-35-5
2,3-Dichlorophenol	576-24-9
3,4-Dichlorophenol	95-77-2
2,4,6-Trichlorophenol	88-06-2
2,3,6-Trichlorophenol	933-75-5

Compounds	CAS Number
2,6-Dibromophenol	608-33-3
2,4,5-Trichlorophenol	95-95-4
2,3,5-Trichlorophenol	933-78-8
2,4-Dibromophenol	615-58-7
2,3,4-Trichlorophenol	15950-66-0
3,4,5-Trichlorophenol	609-19-8
2,3,5,6-Tetrachlorophenol	935-95-5
2,3,4,6-Tetrachlorophenol	58-90-2
2,3,4,5-Tetrachlorophenol	4901-51-3
2,4,6-Tribromphenol	118-79-6
Pentachlorophenol	87-86-5

Table 3. Retention time and selected ion mass.

Compounds (to Derivatization)	RT (min)	Derivatization m/Z	Quantification m/Z
2-Chlorophenol	12.12	181	308
3-Chlorophenol	12.75	181	308
4-Chlorophenol	13.06	181	308
2,6-Dichlorophenol	13.22	181	342
2,5-Dichlorophenol	13.56	181	342
2,4-Dichlorophenol	13.74	181	342
3,5-Dichlorophenol	13.74	181	342
2,3-Dichlorophenol	13.86	181	342
3,4-Dichlorophenol	14.31	181	342
2,4,6-Trichlorophenol	14.68	181	376
2,3,6-Trichlorophenol	14.68	181	376
2,6-Dibromophenol	15.24	181	432
2,4,5-Trichlorophenol	15.49	181	376
2,3,5-Trichlorophenol	15.50	181	376
2,4-Dibromophenol	15.82	181	432
2,3,4-Trichlorophenol	16.07	181	376
3,4,5-Trichlorophenol	16.07	181	376
2,3,5,6-Tetrachlorophenol	16.45	181	376
2,3,4,6-Tetrachlorophenol	17.37	181	412
2,3,4,5-Tetrachlorophenol	17.28	181	412
2,4,6-Tribromphenol	17.70	181	431
Pentachlorophenol	18.95	181	446

4). Sampling and preparation of the water sample

- ***Sampling:*** Sample was adjusted by adding sodium thiosulfate(80mg) and four drop of sulfuric acid(1+5) to a glass bottle in order to remove residual chlorine. The sample was collected about 1 L without any space and airtightly sealed. All the samples were collected in the same manner.. Collect water samples in glass, completely filled with the sample, The sample collected at site was transported in the icebox to make it kept the sample in a refrigerator at a temperature in the range of 2 °C to 5 °C. All samples were analyzed within 7 days.

5). Procedure

- Solid Phase Extraction

a) Water samples are extracted at a pH of less than or equal to 2 with conc. H_2SO_4 1mL per 1L sample. And spike surrogate standard 2-Chlorophenol-d4 20 mg/L 200 uL each standards 1L flask and samples.
b) Multiple SPE tubes were processed simultaneously with a 12-position SPE vacuum manifold. The sample was continuously drawn through the tube using the apparatus shown in Figure 1.
c) Condition : The sorbent was conditioned with 10 mL methanol, followed by 10 mL deionized water.
d) Load : The 1 L sample was continually loaded onto the column using the vacuum manifold apparatus as shown in Figure 1.
e) Elution : The compounds were eluted with 10 mL Acetone.
f) Dehydration : Na_2SO_4 (Sodium sulfate anhydrous, granular) 20 g with filter paper.
g) Extracted samples about 10 mL Acetone were keeping in the -76℃ refrigerator before next step.
h) The phenols are to be determined by derivatization, the extraction solvent was concentrated down to 1 mL using Evaporator. PFBBr derivatization is being performed samples are diluted to a final volume of 2 mL with acetone.
i) PFBBr derivatization procedure.
j) Calibration standards and sample extracts were derivatized using the same procedures.
k) Using the phenol standard mixture stock solutions at 100 mg/L make a composite solution and dilute with acetone to the appropriate concentrations for the calibration range of the analysis.
l) Add 200 µL of calibration standards and sample extracts to 5 mL acetone in a 10 mL graduated concentrator tube with screw caps. Add 100 µL of 5% PFBBr reagent and 100 µL of K_2CO_3 solution to the composite standard.
m) Cap the tubes tightly and gently shake the contents. Heat the tube in a water bath at 60℃ for one hour.
n) After the reaction is complete, cool the solution and concentrate it to 0.2 mL, using nitrogen blowdown.
o) Spike internal standard 2.5-Dibromotoluene 100 mg/L, 100 uL spiking.
p) Add a little hexane and concentrate the solution to a final volume of 1.0 mL.

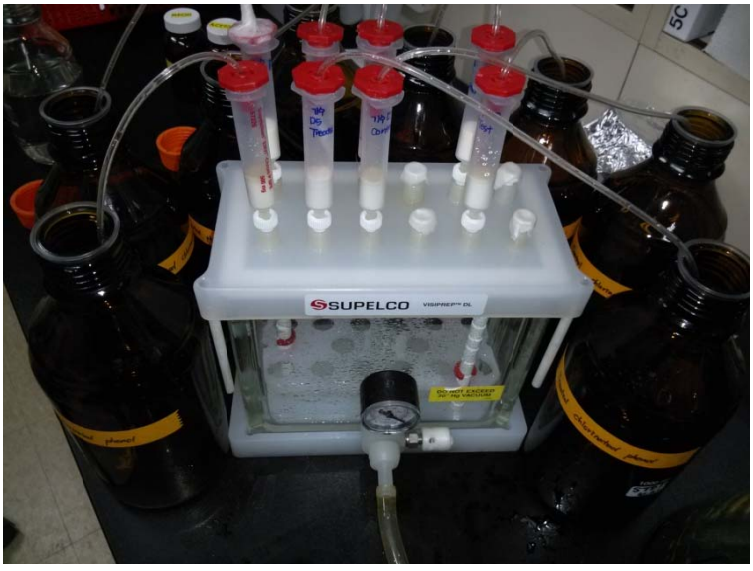


Figure B. Extraction of phenols from sample.

6). Quality Control

- ***Calibration:***

curves, and as a data from manufacturer. or Detection limit wa Initial calibration should be conducted with at least five different concentrations of target analytical standard

- ***Method detection limit :*** The one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration s calculated value that S × 3.14
- ***Precision (%)*** = RSD = S/x × 100 ;
  x : Mean measured value, S : standard deviation
- ***Accuracy (%)*** = x/xi × 100 ; xi : Certified or theoretical value, x : Mean measured value.

****(law data : KTR–Val–PCP_1)***

9. Hydrogen & Oxygen: ISO 6974-3 : 2000

1). Scope and Application

This test method is a way to simultaneously measure Hydrogen and Oxygen among gas created in BWMS. This is the measurement method with Gas chromatograph spectrometry using column.
This test method is applied to analyze Hydrogen, Helium, Oxygen, Nitrogen, Carbon dioxide and Hydrocarbons up to C8 using two packed columns.

2). Equipment and Apparatus

- ***For analysis equipment***: GC(CLARUS 680 GC, PerkinElmer, USA) (Figure A). The details of analysis condition were described in Table 1.

Figure A. Gas chromatograph

Table 1. GC condition for Hydrogen/Oxygen

Variable	Value
GC COLUMN/GAS	6'HayeSep N 60/80 SF, 29'Mol Sieve 13 x 45/60 SF, Argon at 28 mL/min(확인)
GC Injector	Temp : 200 °C
GC Oven	60 °C(isothermal)
Methanizer	Temp : 400 °C
Detector	FID(Temp : 250 °C), TCD(Temp : 200 °C, Ref. 30 mL)

3). Standards

- ***Standard gas :*** Hydrogen(99.9995 %), Oxygen(20.93 %).

4). Sampling

The sample was collected about 10 L(Tedlar bag, BMS, Japan) with gas sampler. All samples were analyzed within 7 days(Figure B).

Figure B. Gas Sampler

5). Procedure

- ***Calibration:*** When making Calibration curve, the minimum quantitation range becomes zero % to verify a linearity of Calibration curve(one point calibration, H_2 : 99.9995 %, O_2 : 20.93 %).

- ***Procedure:***

a) 1 mL of standard gas(H_2) are accurately injected with Gas chromatograph by using 6-port gas sampling valve loop.
b) 1 mL of standard gas(O_2) are accurately injected with Gas chromatograph by using 6-port gas sampling valve loop.
c) After that, two calibration curves have merge to form a new calibration curve.
d) The sample gas(1 mL) are injected with Gas chromatograph.

10.AOX: ISO 9562 : 2004

1). Scope and Application

This test method is a way to simultaneously measure adsorbable organically bound halogens among active materials created in BWMS. The AOX in sample can be analysed by a modified method(dissolved adsorbable organically bound halogens after solid phase extraction in waters with high salt content(SPE-AOX), and then concentrated into adsorption tube(activated carbon) and heated. This is the measurement method with AOX analyzer.

2). Equipment and Apparatus

- ***For analysis equipment***: AOX analyzer(Multi X 2500, Analytik Jena AG, Germany) (Figure A). The details of analysis condition were described in Table 1.

Figure A. AOX analyzer

Table 1. AOX analyzer condition for AOX

Variable	Value
Furnace Temp	950 °C (확인)
Cell Temp	18 °C
Indicator value	15000 ~ 20000
Gas flow	150 mL/min

3). Reagent and Standards

- ***Standard solution :*** 4-Chlorophenol
- ***HNO_3***
- ***HCl(0.01 mol/L)***
- ***H_2SO_4***
- ***$NaNO_3$(0.01 mol/L)***
- ***Na_2SO_3(1 mol/L)***
- ***Adsorption tube(activated carbon)***
- ***Methanol***

4). Sampling and preparation of the water sample

- ***Sampling:*** Sample was adjusted by adding sodium sulfite solution(5 mL) and nitric acid(1 mL) to a glass bottle. The sample was collected about 500 mL without any space and airtightly sealed. All the samples were collected in the same manner. Collect water samples in glass, completely filled with the sample, The sample collected at site was transported in the icebox to make it kept the sample in a refrigerator at a temperature in the range of 2 °C to 4 °C. All samples were analyzed within 7 days.

- ***Preparation of the water sample:*** Filtering of the sample through a filter with en effective pore size of 0.45 µm. Adsorption of organic compounds contained in the sample on styrene-divinylbenzene copolymerized resin. Displacement of inorganic halides by rinsing the resin with acidified sodim nitrate solution. Elution of the resin with methanol, dilution of the methanolic solution with water.

5). Procedure

- ***Calibration:*** The test sample taken for analysis shall have a AOX value within the optimal working range of the instrument, which is generally between 10 µg/L to 300 µg/L. The chloride concentration shall not exceed 1 g/L. It may be necessary to dilute the sample with dilute HNO_3 to achieve a pH < 2 before starting the analysis. When dilution is necessary, do not sue less than 5 mL of the original sample. If the dilution factor is greater than 10, dilute in at least two steps.

a) Dissolve 1, 5, 10 mL of AOX standard solution(1.0 mg/L) in 100 mL volumetric flask and make up to volume with water. The concentration of standard solution is applicable to 10, 50, 100 µg/L.
b) Run the treated test sample through two adsorption columns, vertically mounted in series, at a flow rate of 3 mL/min.
c) The temperature in the combustion apparatus shall be at least 950 °C. Connect the gas supply to the combustion tube and the combustion tube to the absorber. Adjust the gas flow rate to about 150 mL/mim.
d) Transfer the wet activated carbon and the ceramic wool from each column or using the column procedure to the quartz container.
e) Introduce the quartz container into the heated zone of the combustion apparatus equipped with the argentometric measurement device.

6). Quality Control

Check the entire procedure by running a standard solution with a concentration in the middle of the working range of the instrument. The AOX value thus obtained and the theoretical value shall not deviate by more than 10 %(recovery 90 % to 110 %).

Pro. director

Check list of Sampling

Project ID			
Kind of test	Land-based :	■ Basic	■ Final
Test salinity	32 psu	3-32psu	<3psu
Test Cycle			
Prepared by		Checked by	

No.	Item	ea	Use	Remark	Day0	Day1	Day5
					Test date		
1	Bottle 500 mL (Brown G)	1 ea	with label	VOCs/THMs			
	Bottle 125 mL (PE)	2 ea	with label	BrO_3^-, DOC/POC			
	Bottle 500 mL (Brown G)	1 ea	with label	AOX			
	Bottle 1L (Brown G)	1 ea	with label	HAAs, HANs			
	Bottle 1 L (Brown G)	1 ea	with label	Halogenated Phenols			
	Bottle 1 L (G)	1 ea	with label	Br^-/Spare			
2	10 L Vessel	1 ea					
3	15L Vessel	3 ea					
4	2 L Vessel	2 ea					
5	D.W. 20 L	-					
6	Disposable pipette	1 box					
7	Lab tissue	1 box					
8	Laboratory film (Parafilm)	1 roll					
9	Label pen	some					
10	GASTEC (Detector Tube)	1 set		S^{2-} / H_2 / O_3 / Cl_2			
11	GASTEC (Pump)	1 set					
12	S^{2-} kit	1 set					
13	TRO/FRO set	1 set					
14	1, 5 mL Micropipette	Each 1 ea	with Tip				
15	500 mL Mass cylinder	1 ea					
16	200 mL Mass cylinder	1 ea					
17	250 mL Beaker	2 ea					
18	5, 10 mL pipette	Each 10 ea					
19	Pipette Feller	1 ea					

20	50 mL Burette	2 ea					
21	Sodium thiosulfate	1 L	0.1 N				
22	Starch Solution	100 mL					
23	o-Tolidine	1 L					
24	KI	1 ea					
25	Sampling Record	1 ea					
26	Ice box	5 box					
27	Ice	-					
28	Power leader	1 ea					
29	Burette stand	1 ea					
30	Acetic acid	1 ea					
31	NaClO	1 ea					
32	Volumetric flask (100,50 mL)	Each 2 ea					
33	HACH chlorine test kit	1 ea					
34	Tedlar gas sampling bag (5,10 L)	Each 5 ea					
35	Versatile pump (VP-18T)	1 ea					
36	Vacuum pump	1 ea					
37	Water collection bottle	1 ea					
38	H_2SO_4	1 ea		DOC/POC, VOCs/THMs, Halogenated Phenols			
39	HCl	1 ea		HAAs, HANs			
40	HNO_3	1 ea		AOX			
41	Ethylenediamine	1 ea		BrO_3^-			
42	Na_2SO_3	1 ea		AOX			
43	$Na_2S_2O_3$	1 ea		VOCs/THMs, Halogenated Phenols			
44	NH_4Cl	1 ea		HAAs, HANs			

Pro. director

Sampling Records

Receipt No.		Client	
Receipt Date		Client address	
Item		Sampling Place	
Lot. No.		Sampling Date	
Sampling Method	ISO 5667-3/ Test method	Sampling collector	(sign)
		Sampling Manager	(sign)

Test item	Environment condition	Applied Standard	Description (container,volume; preservation)	Data / Remark
TRO	Weather : **Sun / Cloud / Rain**	ISO 7393-2/EPA 330.5/ Standard Method 21th 4100-Cl G DPD Colorimetric Method	In site	* Table1
FRO		ISO 7393-2/Drinking Water Quality Standard and Test ES 05310.1, Korea	In site	
O_3		ATI Q45H-64-3/ Standard method 21th Edition, 4500-O -B	In site	
ClO_2	Atmospheric temp. : ,	ATI Q45H-65-3/ Standard method 21th Edition, 4500-ClO -B	In site	
S^{-2}		Standard method 21th Edition, 4500- S^{-2}-D, Dector tube	In site	
Bromate		ISO 15061	PE, 125 mL; Ethylenediamine 5 mg	Y / N
DOC / POC	Humidity : %	ISO 8245	PE, 125 mL ; H_3PO_4 2 drop	Y / N
Density		ISO 15212-1 Oscillation-type density meters-Part 1	Spare ,G[2], 1 L	Y / N
THMs / VOCs	Atmospheric pressure : mbar	US EPA 524.2	B.G[1] , 500 mL; $H_2SO_4$2 drop+ $Na_2S_2O_3$ $5H_2O$ 4 mg	Y / N
AOX		ISO 9562	B.G, 500mL; Na_2SO_3[3]5.0mL +HNO3 1.0 mL	Y / N
HANs / HAAs		US EPA 551.1/US EPA 552.2	B.G, 1 L; NH_4Cl 10 mg +HCl 4 Drops	Y / N
Hologenated Phenols	Sample temp.: ,	US EPA 8041A	B.G, 1 L*2; ; $H_2SO_4$2 drop+ $Na_2S_2O_3$•$5H_2O$ 4 mg	Y / N
Spare		ATI A14/11, ISO 6974-3	G, 1 L	Y / N
H_2 / O_2 gas	Sampling time :	Detector tube	In site	
O_3 gas	Hr min	Detector tube	In site	
Cl_2 gas			In site	

Witness(***KOMERI***)	Position	Project manager / Project researcher	(sign)

Hand over	Analyst	**Ji hyun, Lee**	**Young keun, Im**	**Sun chool, Hwang**	**Jun ho, Park**	**Jin hoon, Do**
		Date:	Date:	Date:	Date:	Date:
		Sign:	Sign:	Sign:	Sign:	Sign:

B.G[1] : Brown Glass, G[2] : Glass, Na_2SO_3[3] : 126 g Na_2SO_3 dissolved in distilled water

* Table 1. The concentration of TRO, FRO

	1	2	3	4	5
TRO					
FRO					

1. Element : **Dichloromethane**

2. Spkied Level : **mg/L**

3. Test Method :

4. Instrument :

5. MDL : ① The concentration value that corresponds to the instrument S/N ratio in the range of 2.5 to 5

② Standard deviation as calculated from 7 repeated measurements from blank water spiked with **mg/L** concentration

6. Accuracy Accuracy is estimated as the difference between the measured and target values of performance evaluation samples at the lower concentration range, and as the percent difference at the higher concentration range.

7. Precision Precision is estimated as the percent relative standard deviation of repeated measurement

8. Measurement

Repeat	Conc.(mg/L)	xi-x	(xi-x)2	Accuracy(%)
1				
2				
3				
4				
5				
6				
7				
Mean (x)				
Σ(xi-x)2				

9. Standard deviation (s) $s = \sqrt{\frac{\sum(x_i - x)^2}{n-1}}$

s = 0.0000

10. MDL MDL= $T_{(n-1,\ 1-\alpha = 0.99)} \times s$

$T_{(n-1,\ 1-\alpha = 0.99)}$: the student's t value appropriate for 99% confidence level and a standard deviation estimate with n-1 degrees of freedom ; if, n=7, t-statistic value is 3.14

MDL=	**mg/L**

10. Precision

11. Check

Validation item	value	unit	Acceptable range
a. Calibration (r^2)		-	: >0.99
b. MDL		mg/L	-
c. Accuracy		%	: 70~130 %
d. Precision		%	: < 20 %

Standard Operation Procedures (SOP)

for

Aquatic Toxicity Test

Appendix 3.4.3

Contents of APPENDIX 3.4.3

1. Standard Operation Procedure list

1. Standard Operation Procedure list

Major SOP (Standard Operation Procedure) list for this study is shown as follow:

.1 SOP of TP (Test Procedures)

a. SOP#TP-001: Phytoplankton, Growth Inhibition Test.
b. SOP#TP-006: Fish, Acute Toxicity.
c. SOP#TP-007: Fish, Short-term Toxicity Test on Embryo and Sac-fry Stage.
d. SOP#TP-008: Phytoplankton, Inoculation Methods.
e. SOP#TP-009: Phytoplankton, Counting of Cell numbers.
f. SOP#TP-010: Fish, Measurement of Length and Weight with Larva and Juvenile.
g. SOP#TP-011: Test Water, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.
h. SOP#TP-012: Measurement of Chlorophyll a and Phaeopigment by fluorometric analysis.
i. SOP#TP-013: Rotifer, Acute Toxicity Test.
j. SOP#TP-014: Rotifer, Population Growth Test.

.2 SOP of TO (Test Organisms)

a. SOP#TO-001: Phytoplankton, Isolation and Culture.
b. SOP#TO-005: Fish, Transportation and Laboratory Culturing.
c. SOP#TO-007: Rotifer, Cyst Hatching Method.

.3 SOP of TE (Test Equipments)

a. SOP#TE-002: Water Quality Checker

.4 SOP of SP (Statistical Procedure)

a. SOP#SP-101: Toxicity data analysis with Toxcalc software
b. SOP#SP-102: Linear regression analysis with Excel program

2. SOP of TP (Test Procedures)

SOP#TP-001

(Lastest revised January 2011)

PHYTOPLANKTON, GROWTH INHIBITION TEST

This SOP based on principle of the ISO 10253, Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum* that was to determine the effect of an substance (or effluence/receiving water) on the growth of marine microalgae.

DEFINITIONS

Cell density is number of cells per unit volume of medium (x cells/ml).

Specific Growth rate is proportional rate of increase in cell density per unit of time.

Growth medium is mixture of sea water and nutrients which is used for pre-culture and controls.

Test medium is mixture of sea water, nutrients (growth medium) and test material in which algal cells are incubated.

Test batch is mixture of seawater, nutrients and test material (test medium) inoculated with algae.

Control is mixture of seawater, nutrients (growth medium) without test material, inoculated with algae.

Effective concentration (EC*x*) is concentration of test substance which results in an *x*% reduction in specific growth rate relative to the controls.

STEPS	COMMENTS
1. Preparation	
1.1 Apparatus	
.1 Apparatus sterilized Nalgene filter set, 0.2 ㎛ membrane filter, Forceps for cover glass/paper, Silicon hose, f/2 marine water solution, Flask (250mL, 18 ea), 100 mL Mass flask, Bio-steristopper (18 ea), 10 mL and 1 mL Micro pipet, 10 mL and 1 mL pipet tips	1.1.1 The flasks are used as test chamber. But the test chamber can substitute flask by test tube. If the test tube is chosen as test chamber, volume of the test solution should be recalculated, depending on the volume of the test tube.
.2 Non-sterilization Stereomicroscope (CKX 31, Olympus), Clean-bench, Gas burner (or gas torch), Incubator, Vacuum pump, 1L Beaker, Inverted Microscope, Water quality checker (WQC-22a, Japan)	

STEPS	COMMENTS
1.2 Test Water	
.1 Chemical substance: For making stock solution, the chemical substance was dissolved in the natural seawater or artificial seawater making	
.2 Effluence or receiving water: For making stock solution, the effluence or receiving water diluted in the natural seawater or artificial seawater using control seawater filtered with 0.45 μm membrane filter.	1.2.2 see SOP#TP-011
1.3 Dilution water Dilution water for the toxicity test is used uncontaminated natural filtered seawater (0.45 μm membrane filter) or artificial seawater.	1.3 Dilution water is used to make a test solution
2. Methods	
2.1 Preliminary Test	
.1 The concentrations of the test substance range in five concentrations	2.1.1 Preliminary test is range-finding test. For the main test (final definitive test), the concentration range in which effects are likely to occur may be determined on the basis of results from range-finding test.
.2 The procedure of preliminary test is equal to section 2.2.1. The control and replicates of each concentration in the preliminary test are not necessary.	
.3 The algal cell density in each flask is determined at least daily during the test period. Measurement of cell density is done by manual cell counting by microscope.	2.1.3 See SOP#TP-009.
2.2 Main Test	
2.2.1 Procedures	
.1 An inoculum culture, the test alga in the test medium is prepared on 3 days before start of the test.	
.2 Make the 6 concentration of test solution including control.	2.2.1.2 For the final definitive test at least six concentrations including the control, arranged in a geometric series with a factor not exceeding 3.2, are selected. When a solvent is used to solubilize the test substance, additional control containing the solvent at the same concentration as used in the test culture must be included.

STEPS	COMMENTS
.3 Prepare 3 sterilized flasks in each test concentration.	2.2.1.3 The test design includes three replicates at each test concentration.
.4 Measure the cell density of the test alga in exponential growth phase and calculate the inoculum volume.	2.2.1.4 Microscopic observation is performed to verify a normal and healthy appearance of the inoculum culture
.5 Place on all sterilized apparatus of the test in clean-bench after spraying the 70% alcohol.	
.6 Connect the Nalgene filter-set in clean-bench to one silicon hose with 0.2 m syringe filter.	2.2.1.6 The syringe filter can prevent invasion of bacteria from vacuum pump.
.7 And then connect the other silicon hose to the vacuum pump out of clean-bench and to the 0.2 m syringe filter from Nalgene filter-set.	
.8 Turn on the UV lamp on the clean-bench for 10 minute.	
.9 Place all flasks filled with each concentration of the test solution in clean-bench after turning off the UV lamp.	
.10 Filter the 350 mL of the test solutions of each test concentration through 0.2 m membrane filter.	2.2.1.10 The necessary volume for the test is only 300 mL (100 mL test solution 3 replication). However, residual test solution is used to rinse the test flask with it.
.11 Fill with the 50 mL test solution sterilized by filter in 100 mL mass flask and then put the test solution sterilized on the line of the mass flask after adding the 2 mL of the f/2 medium.	
.12 Place the flask of inoculum culture from incubator in the clean-bench after spraying 70 % alcohol. And then open the flask after sterilizing top of the flask containing test organism by gas burner.	
.13 Inoculate test alga with test solution in the each flask that initial cell density is finally 3×10^3 cells/mL.	
.14 Cap the bio-steristopper after inoculation	
.15 Place the test cultures in the incubator controlled test condition after labeling information of the test that is the concentration, replication and test date.	2.2.1.15 The test condition is showed Appendix I. The test flasks are shaken gently and place the flask randomly and reposition them daily in the incubator.
.16 Determine the algal cell density in each flask at least daily during the test period and record it on the log sheet.	2.2.1.16 See SOP#TP-009. Test duration is normally 72 hours. However, shorter or longer test duration is used provided that all validity criteria can be met. Measurement of cell density is done by manual cell counting with

STEPS	COMMENTS
	microscope and by estimating the chlorophyll a concentration in the test chamber (see SOP#TP-012). Microscopic observation is performed to observe any abnormal appearance of the algae at the end of the test.
2.2.2 Measurement of test water 2.2.2.1 Measure the pH of the solutions at the beginning and at the end of the test.	2.2.2.1 See SOP#TE-006. The pH of the control medium does not increase by more than 1.5 units during the test.
2.2.3 Data analysis **2.2.3.1 Average growth rate** The average growth rate for specific period is calculated as logarithmic increase in the cell density from the equation for each single flask of control and treatment [1]: $\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}(day^{-1})$ ----------[1] Where: μ_{i-j} is the average specific growth rate from i to j; X_i : is the cell density at time i; X_j : is the cell density at time j.	2.2.3.1 Calculate the average specific growth rate over the entire test duration (normally days 0-3), using the nominally inoculated cell density as the starting value rather than a measured starting value, because in this way greater precision is normally obtained. Assess also the section-by-section growth rate, calculated as the specific growth rates for each day during the course of the test (days 0-1, 1-2 and 2-3) and examine whether the control growth rate remains constant.
2.2.3.2 Inhibition of the growth rate Calculate the percent inhibition of growth rate for each treatment replicate from equation [2]: $\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$ ----------[2] Where: $\%I_r$ is percent inhibition in average of specific growth rate; μ_C is mean value for average of specific growth rate (u) in control group; μ_T: is average of specific growth rate for treatment replicate.	2.2.3.2 When solvents are used to prepare the test solutions, the solvent controls rather than the controls without solvents is used in calculation of percent inhibition.

STEPS	COMMENTS
2.2.3.3 Yield The percent inhibition in yield ($\%I_y$) is calculated for each treatment replicate as follows [3]: $$\%I_y = \frac{Y_C - Y_T}{Y_C} \times 100$$ ---------[3] Where: $\%I_y$ is percent inhibition of yield; Y_C is mean value for yield in the control group; Y_T is value for yield for the treatment replicate.	2.2.3.3 Yield calculated as the cell density at the end of the minus the starting cell density for each single flask of controls and treatments. For each test concentration and control, calculate a mean value for yield along with variance estimates.

APPENDIX Ⅰ

Test conditions & acceptability criteria

Test parameter	Conditions
Test type	Static
Temperature	22 ± 1 ˚C
Light quality	“Cool white” fluorescent lamp
Light intensity	3,000 lux
Photoperiod	Continuous illumination
Test chamber size	15 mL
Test solution volume	10 mL
Renewal of test solutions	None
Initial cell density in test chambers	3,000 cells/mL
No. replicate chambers per concentration	3
Shaking rate	Twice daily by hand
Aeration	None
Dilution water	Filtered seawater (Control seawater) by 0.2μm membrane filter
Test concentrations	Effluents: 6 including receiving water 100.00% and a control
Test dilution factor	Effluents: 0.5
Test duration	72 hours
Endpoint	Growth inhibition
Test acceptability criteria	Specific growth rate in the control: ≥ 0.92 day^{-1} Variation coefficient of the control specific growth rate during the whole test period: ≤ 7%
Precision object	Reference toxicant: potassium dichromate 72h EC50 value: 1.4~3.6 mg/L

SOP#TP-006
FISH, ACUTE TOXICITY TEST

This SOP is common procedure for acute aquatic toxicity test of fish. This based on OECD GUIDELINE FOR TESTING OF CHEMICALS, 203. (17th July 1992).

DEFINITIONS

Semi-static test is a test with out flow of solution, but with occasional batchwise renewal of the test solution of the test solution after prolonged.

LC_{50} is the median lethal concentration, i.e. that concentration of the test substance in water which kills 50 per cent of a test batch of fish within a particular period of exposure (which must be stand)

STEPS	COMMENTS
1. Materials	
1.1. Test organisms (fish)	
1.2. Sampling water	
1.3. Test tanks (30L glass cubic aquarium)	1.3. Tanks made of chemically inert material and of a suitable capacity.
1.4. Temperature control system (Constant temperature room)	
1.5. Water quality checker	
1.6. Aeration equipments	
1.7. General Laboratory equipment.	
2. Methods	
2.1. Preparation	
2.1.1. Fish must be obtained and held in the laboratory for at least 2weeks (14days) before used for testing.	2.1.1. Transportation and laboratory culturing of fish. **(SOP#TO-005)**
2.1.2. Sampling water for test and filtered, keep suitable temperature for test.	2.1.2. Effluent and receiving water sampling, sample handling, and preparation for toxicity tests. **(SOP#TP-011)**
2.1.3. Setting at least five concentrations in a geometric series.	

STEPS	COMENTS
2.1.4. Test substance dissolved to sampling water and make test water that number of each test concentration.	
2.1.5. In the Constant temperature room, install a 30L glass cubic water tank for test by 3 replications and attach a label.	
2.1.6. Fill the test water in to tanks considering the test organism (fish) weights.	2.1.6. Maximum loading of 1.0 g fish/L for static and semi-static tests is recommended; for flow-through systems higher loading can be accepted.
2.1.7. The dissolved oxygen concentration must have been at least 60 per cent of the air saturation value during the test.	2.1.7. The water temperature must not differ by more than 1 between test tanks at any one time during the test.
2.1.8. To each water tank, put a fish and close the cap of a tank for prevent seawater and test substance vaporization.	2.1.8. Aeration can be used provided that it does not lead to a significant loss of test substance.
2.2. Observation	
2.2.1. The fish are inspected at least after 24, 48, 72 and 96 hours. Fish are considered dead if there is no visible movement and if touching of the caudal peduncle produces no reaction.	2.2.1. The mortality in the control should not exceed 10 per cent at the end of the test.
2.2.2. Dead fish are measured wet weight and length, removed when observed and mortalities are recorded.	2.2.2. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.).
2.3. Renewal	
2.3.1. For semi-static, test water renewal once a two days.	2.3.1. Preferably it should be at least 80 per cent of the nominal concentration during the test.
2.4. Measurement of water conditions	
2.4.1. pH, DO, Salinity, and Temperature of concentrations during the test period were	2.4.1. Water Quality Checker (WQC-022A) **(SOP#TE-002).**

STEPS	COMENTS
measured and recorded daily. 2.4.2. In semi-static systems water condition should be measured prior to and after water renewal.	
2.5. Statistical procedures	
2.5.1. The aim is to obtain a quantitative concentration-response (Mortality) relationship by a probit analysis. For each response variable to be analyzed, use the concentration-response relationship to calculate point estimates of EC50 and LC50. 2.5.2. Record the results to report, and finish the test.	2.5.1. **(SOP#SP-101)**.

SOP#TP-007
FISH, SHORT-TERM TOXICITY TEST ON EMBRYO AND SAC-FRY STAGE

This SOP is based on principle of the OECD guideline 212 (Fish, Short-term Toxicity Test on Embryo and Sac-fry stage). The embryo and sac-fry stage of fish are exposed to a range of concentration of the test substance dissolved in water. Test is begun by placing fertilized eggs in the test chambers and is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in the controls. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed concentration (LOEC) and hence the no observed concentration (NOEC). Alternatively, they may be analyzed using a regression model in order to estimate the concentration that would cause a given percentage effect (i.e. LC/EC_x where x is a define % effect).

DEFINITIONS

LOEC (Lowest observed effect concentration) is the lowest test concentration of test substance at which the substance is observed to have a significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

NOEC (NO observed effect concentration) is the test concentration immediately below the LOEC.

Test substance is crude soluble chemical substance, effluence and/or receiving water to effect on survival, normality, reproduction or mobility of the test organism.

Test solution is prepared for the test by dissolved test substance on the dilution water and/or natural seawater, by diluted effluence or receiving water on the dilution water and/or natural seawater.

STEPS	COMMENTS
1. Preparation	
1.1 Test Organism (Fertilized eggs) *Paralichthys olivaceus* *Sebastes* sp. etc.	1.1 Fertilized eggs of fish less than 8 hours are used as test organism. The eggs fertilized within 8 hours are obtained from commercial supplier.
1.2 Apparatus 2 L volume of test chamber with 200 μm mesh, 10 L aquarium, Petridish, Spuit, Stereomicroscope (SZ-51, Olympus),	1.2 Test chamber should be modified by volume of aquarium. Scheme is showed in the ‘Appendix II’.
1.3 Test water	
.1 Chemical substance:	

STEPS	COMMENTS
For making stock solution, the chemical substance was dissolved in the natural seawater or artificial seawater making	
.2 Effluents and/or receiving seawater: For making stock solution, the effluent or receiving seawater diluted in the natural seawater or artificial seawater using control seawater filtered with 3 μm CP Filter.	1.3.2 See SOP#TP-011.
.3 Dilution water for the toxicity test is used uncontaminated natural filtered seawater (3 μm CP Filter) or artificial seawater.	1.3.3 Dilution water is used to make concentration of the test solution.

2. Methods

2.1 Procedures

.1 Make the 6 concentration of test solution including control.	2.1.1 For the final definitive test at least six concentrations including the control, arranged in a geometric series with a factor not exceeding 3.2, are selected. When a solvent is used to solubilize the test substance, additional control containing the solvent at the same concentration as used in the test culture must be included. Experiment conditions and acceptability criteria are showed in the 'Appendix I'.
.2 Prepare 3 of 2 L test chamber with 200 μm mesh per test concentration labeled test date, concentration and replicate number.	
.3 Fill with test solution (10 L) in each test vessel (10 L aquarium).	
.4 Divide 30 individuals in each test chamber using spuit modified to the fertilized eggs.	

STEPS	COMMENTS
.5 Place the test vessels at 20 ˚C in the constant room.	2.1.5 Disolved Oxygen concentration should be maintained more than 5 mg/L.
.6 Connect air hose to the aeration equipment. For the gentle aeration, the cock on the aeration pipe is adjusted.	
.7 check and record observation items each 30 minutes during the first three hours after the beginning of test.	2.1.7~8 Observation items are showed in the ‘Appendix III’.
.8 Second, daily check and record observation items for 7 days.	
.9 Dead eggs, embryos and larvae are removed after record.	
.10 Renewal of the test solution is 3 times per a week.	
.11 Measure the length and weight of the larvae under the stereomicroscope at the end of the test.	2.1.11 Total length and dry weight is measured.

2.2 Measurement of test water

2.2.1 Measure the pH and DO of the solutions every day. The old and fresh test solution should be measured when the test solution is exchanged by fresh solution.

APPENDIX Ⅰ

Experiment conditions & acceptability criteria

Test parameter	Conditions
Test type	Semi-static
Temperature	20 ± 1 ˚C
Light quality	Cool white fluorescent lighting
Light intensity	2,000 lux
Photoperiod	16 h light: 8 h dark
Test camber size	2 L
Test solution volume	1.8 L
Renewal of test solutions	3 times per a week
Age of test organisms	Less than 8 hours
Number of individuals per test chamber	30
Replicate test chambers per concentration	3
Feeding regime	None
Cleaning	Exchange the test water
Aeration	To maintain DO (5.0 mg/L)
Dilution water	Filtered seawater (3 μm CP Filter, Chisso Filter)
Test concentration	6 including 100 % effluents and a control
Dilution factor	Effluents: 0.5
Test duration	7 days
Endpoints	Survival
Test acceptability criteria	- The water temperature change: less than ± 1.5 ˚C - Overall survival of fertilized eggs in the controls: more than 70 %

APPENDIX II

Observation items

Stage of embryonic development: the embryonic stage (i.e. gastrula stage) at the beginning of exposure to the test substance should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleared.

Hatching and survival: observations on hatching and survival should be made at least once daily and numbers recorded. It may be desirable to make more frequent observations at the beginning of the test (each 30 minutes during the first three hours), since in some cases, survival times can be more relevant than only the number of deaths (e.g. when there are acute toxic effects). Dead embryos and larvae should be removed as soon as observed since they can decompose rapidly. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

- for eggs: particularly in the early stages, a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;
- for embryos: absence of body movement and/or absence of heart-beat and/or opaque discoloration in species whose embryos are normally translucent;
- for larvae: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque colouration of central nervous system and/or lack of reaction mechanical stimulus.

Abnormal appearance: the number of larvae showing abnormality of body form and/or pigmentation, and the stage of yolk-sac absorption, should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test vessels on death.

Abnormal behaviour: abnormalities, e.g. hyperventilation, uncoordinated swimming, and atypical quiescence should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data i.e. provide information on the mode of toxic action of the substance.

Length: at the end of the test, measurement of individual lengths is recommended; standard, fork or total length may be used; if however, caudal fin rot or fin erosion occurs, standard lengths should be used. Generally, in a well-run test, the coefficient of variation for length among replicates in the controls

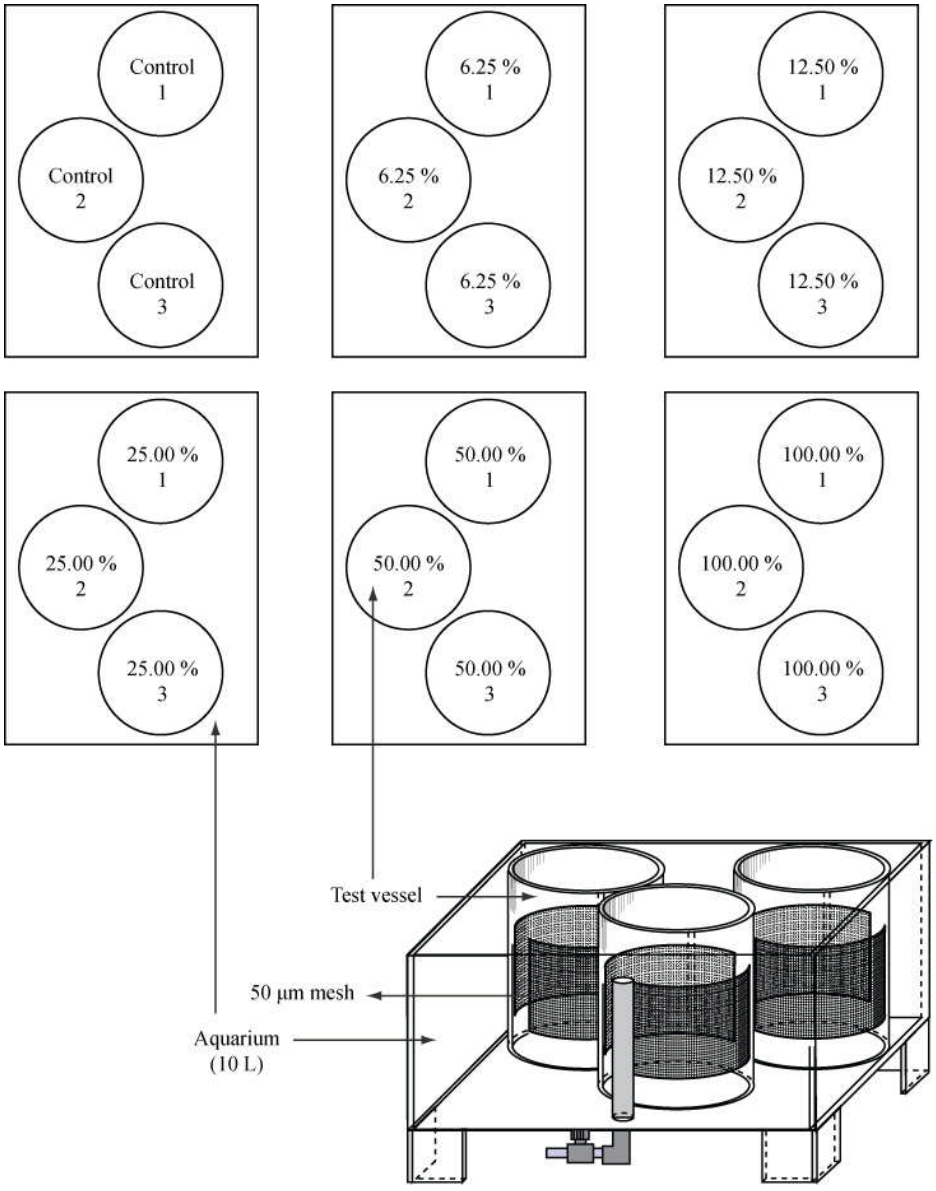
should be < 20%.

Weight: at the end of the test, individual weights can be measured; dry weights (24 hours at 60 ºC) are preferable to wet weights (blotted dry). Generally, in a well-run test, the coefficient of variation for weight among replicates in the controls should be < 20%.

APPENDIX III

Application Site

Application site for the chronic toxicity test with the fertilized eggs of fish was shown as follow.



SOP#TP-008
PHYTOPLANKTON, INOCULUM METHODS

This SOP is a common procedure for transferring established cultures into new culture medium. Although many variations exist in different laboratories, this provides a general procedure for proper sterile technique (reference: Algal Culturing TECHNIQUES. 2005 chapter 5).

STEPS	COMMENTS
1. Materials	
1.1 Culture Organism	
1.2 Apparatus Clean bench, sterilized instrument (10mL pipette, 10mL pipette tip, 250mL erlenmeyer flasks), torch, 70% Ethanol	
2. Methods	
2.1 Preparation	
2.1.1 Wear protective clothing is that is sterilized by UV light.	2.1.1 If clothing is maintained inside the room, then carefully do this after turning off the UV lamp and entering the room.
2.1.2 Turn off the UV sterilizing light in the room, open the door using a minimum opening, enter the room with slow motion, and gently close the door.	
2.1.3 Turn on the flow hood air circulator and then turn off the UV sterilizing light in the clean bench hood.	
2.1.4 Wipe the working surface with 70% Ethanol.	
2.1.5 Place the torch in the center.	
2.1.6 Place the 1mL pipette or 1mL pipette tips on the most left side. Spray and wipe the surface of the container with 70% ethanol.	
2.1.7 Place the Erlenmeyer flasks on the left side.	
2.1.8 Place the container for discarding used pipettes outside the 40-cm circle, on the most right side of the working area.	

STEPS	COMMENTS
2.1.9 Make sure that all inoculation vessels are correctly labeled.	
2.1.10 After organizing all materials, spray hands with 70% ethanol(or put on sterile disposable glove).	2.1.10 Sterile manipulation can now begin; make sure to stay within the 40-cm circle around the torch.
2.2 Inoculum	
2.2.1 The pipette tip put into the pipette.	
2.2.2 Handle the pipette in your right hand near the torch and slowly pick up the cell culture vessel with your left hand.	
2.2.3 Bring the cell culture vessel to your right hand, and using your palm and small finger of the right had while still holding the pipette, remove the silistoper from the vessel.	2.2.3 The pipette should remain close to the torch and should not touch anything.
2.2.4 With the left hand, briefly flame the opening of the cell culture vessel while slowly rotating the lip of the vessel in the flame at an angle of at least 45 degrees.	2.2.4. Avoid breathing into the vessel if the clean bench window is not pulled down. Do not flame plasticware.
2.2.5 With the left hand, move the vessel slowly away from the flame but keep it oriented at a 45 degree angle to reduce the possibility of contamination.	
2.2.6 Slowly insert the tip of the pipette into the pipette by carefully controlling the pressure exerted on the top, collect quantitative volume of cell suspension.	2.2.6 To avoid any risk of spilling or dripping.
2.2.7 After drawing up the appropriate amount of cell suspension, the pipette is slowly removed while avoiding contact with the vessel.	
2.2.8 Flame the mouth of the vessel again, using a rotating motion.	2.2.8 Do not touch the pipette against anything.
2.2.9 Using your left hand, slowly bring the vessel to the sillistoper that has been maintained between the small finger and palm of the right.	2.2.9 For larger silistopers that cannot be held during manipulation, the silistoper is placed on the working area and flamed before replacement.

STEPS	COMMENTS
2.2.10 Replace the silistoper, always being careful that the pipette dose not touch anything.	2.2.10 Be careful not to bring the pipette too close to the flame, because the heat may kill the cells.
2.2.11 Return the capped vessel to its previous position. Your left hand is now free.	2.2.11 It is good practice to place the drawn vessels in a new location so that accidental reinoculation dose not occurs.
2.2.12 Slowly move your left hand to the vessel that will be inoculated.	
2.2.13 Using the same procedure described above, open the new vessel, flame the opening, and insert the pipette into the new vessel without touching the mouth.	
2.2.14 Slowly discharge the cell suspension into the vessel and carefully remove the pipette.	2.2.14 If the tip of the pipette is placed below the surface, then avoid bubbling into the liquid.
2.2.15 Flame the mouth of the vessel and replace the silistoper as described above, using precautions as before.	
2.2.16 The newly inoculated vessel, with silistoper attached, should be placed onto the bench again.	
2.2.17 Discard the used pipette in the storage container, using caution to avoid any spillage of cell suspension onto the bench.	2.2.17 If ant spill has occurred, then wipe up the liquid with a Kimwipe or towel, spray the area 70% ethanol, and wipe the surface again with a new tissue paper. If wearing gloves, then change to a new pair of sterile gloves; otherwise, spray hands with 70% ethanol.
2.2.18 Bring the pipette to the discard container. With the free left hand, pick up the pipette with your thumb and index finger at the cotton-plugged end of the pipette, and carefully remove the pipette bulb with your right hand.	
2.2.19 With your left hand, place the pipette in the container, making sure that the tip is well immersed in the water. The working area is back to its initial stage and ready for the next transfer process.	

STEPS	COMMENTS
2.2.20 Once all transfers are completed, turn off torch, remove all the materials from the working place, and wipe the surface with 70% ethanol.	
2.2.21 Switch off the ventilation system and turn on the UV lamp of the hood. Exit the airlock room by carefully opening and closing the door. Turn off the room light and turn on the UV lamp for the room.	
2.2.22 Remove your protective clothing and place it in the UV cabinet for sterilization. Exit the air lock room carefully and switch on the UV sterilization of this room.	
2.2.23 In the most case, clean shoes or slippers are recommended for the clean area, and these should be changed when entering and exiting the clean area.	2.2.23 A sticky pad or carpet placed on the floor at the door location is very helpful to keep dirt from clean areas; dirt on shoes is perhaps the primary source of contamination entering a room, where later it may become airborne.
2.2.24 Appropriate signs should be in place to ensure a safe work area. Custodians should be generally prohibited from servicing sterile facilities unless informed and instructed.	

SOP#TP-009
PHYTOPLANKTON, COUNTING OF CELL NUMBERS

This SOP is based on principle of the chapter 16, Counting cells in Cultures with the light microscope, in Algal Culturing Techniques (2005).

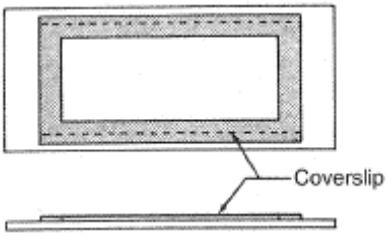
STEPS	COMMENTS
1. Materials	
1.1 Test Organism	
1.2 48 hole plate, gas-torch, 1 mL auto-pipet and tip, lugol solution, filtered seawater (0.45 μm), microscope	
2. Methods	
2.1 Preparation	2.1 Cell unit: cells/mL
.1 Place the 1mL pipet and tip sterilized by autoclave and 48 hole plate disinfected by 70% ethanol in the clean-bench.	
.2 Close the window on the clean-bench and turn on the UV (Ultra-violet) sterilizing light for 20 min and then turn off it.	2.1.2 See “SOP#TE-006”
.3 Spray and wipe the 70% ethanol on the surface of test flasks or test tubes from incubator.	
.4 Spry hands with 70% ethanol.	
.5 Place the test flasks or test tubes in the clean-bench. Flame the cap and mouse of test vessels. And open the cap of test flasks or test tubes.	2.1.5~6 The transfer of test organism should be conducted one by one.
.6 Transfer 1 mL test organism to one of 48 hole plate 1 mL pipet with tip. Close the cap and label on the 48 hole plate.	
.7 Carry out test flasks or test tubes from clean bench and replace it in the incubator.	
.8 Prepare the fixation collected test organism.	

STEPS	COMMENTS
2.2 Fixing and dividing	
.1 Resuspend the samples in the 48 hole plate using 1 mL auto-pipet.	2.2 This diving method is an example that is a kind of the dilution technique (dilutions of 1:2). If the cell concentration is too high, serial dilution should be necessary, e.g. repeated serial dilution of 1:10. Therefore, dilution factor must be recorded because it is different depending on the cell concentration.
.2 Transfer 500 µL resuspended sample in the next hole.	
.3 Add the 100 µL Lugol solution.	
.4 Add the 400 µL filtered seawater (0.45 µm)	
.5 Place the 500 µL of specimen on the Sedgwick-Rafter chamber and settle for 5 minutes.	2.2.5 Choice of counting device depends upon cell density, the size and shape of the cells or colonies being counted, and presence and amount of extra cellular thread, sheaths, or dissolved mucilage. See “Appendix I”.
2.3 Counting	
.1 Count the cells number and observe the cell appearance.	2.3.1 To count the whole Sedgwick-Rafter chamber, position it so the Whipple square is in one corner of the counting chamber, e.g., the upper left as seen through the microscope. Slowly move the stage horizontally (left to right), tallying the organisms as they pass the leading boundary (right side) of the square, until the Whipple square is in the upper-right corner (Count the organisms that are cut by the lower boundary; they will be ignored on the next sweep, when they will lie on the upper boundary). Move the stage vertically so that the Whipple square has (apparently) moved down by its own width (A particle or mark on the glass of the chamber, on or near the bottom of the Whipple square, can be used as an index). Then sweep horizontally from right to left, again tallying organisms. Ignore organisms that lie on the upper boundary of the square. Repeat until the whole chamber has been covered. The total count is the algal density in the chamber as either cells/mL or colonies/mL.
.2 Record result of the cell count and dilution factor.	2.3.2 Total cell density (cells/mL)= counted cell number × dilution factor

APPENDIX Ⅰ

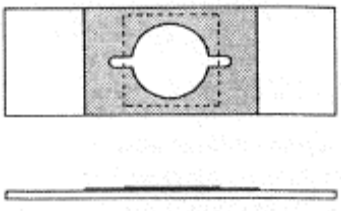
Microscope Counting Devices

1. The Sedwick-Rafter Counting Slide



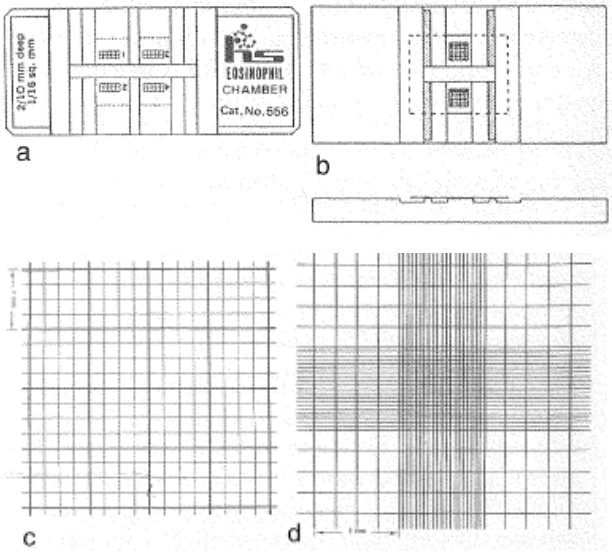
The chamber is without rulings and is rectangular (50 × 20 mm), 1mm deep, of area 1,000 mm^2 and volume 1.0 mL. With no. 1 $^1/_2$ coverslips, most ×20 N.A. 0.5 objectives can be used, permitting magnification to ×500. The largest phytoplankters can be held in this slide, and many species as small as 10 μm can be recognized in it. It is best suited to large and relatively scarce organisms, which can be detected in the concentrate at just a few per milliliter. A concentration of 10^4 cells/mL in the concentrate yield *ca*. 5 cells/Whipple field at ×125 magnification.

2. The Palmer-Maloney slide



The chamber is without rulings and is circular, of diameter 17.9 mm, depth 400 μm, area 250 mm^2, and volume 0.1 mL. It has a loading channel (slot) on each side. High-dry objectives can be used. Cells (of some species) as large as 150 μm will enter and be reasonably well-distributed in this chamber. Even very small phytoplankters can be detected in it. Species in the concentrate at more than 10/mL should at least be detected; 10^4 cells/mL yield *ca*. 2 cells/Whipple field at ×125 magnification.

3. Hemacytometer, 0.2 mm Deep

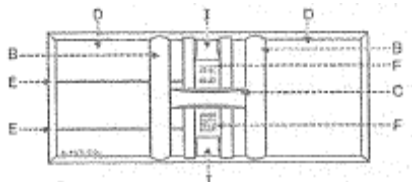


The Speirs-Levy eosinophyll counter has four separate chambers, each having 10 squares each (a). The squares have a modified Fuchs-Rosenthal ruling (c); each 1-mm square is divided into 16 250-μm square. Total volume in all 40 1-mm squares is 0.008 mL; thus, a sample having 10^4cells/mL will yield ca. 80cells total (two cells per 1-mm square). Cell concentrations in the range of 5 × 10^3/mL can at least be estimated and certainly detected. Objectives of 20× can be used with thin coverslips. There are also two chamber hemacytometers 0.2

mm deep (b). Each chamber consists of 16 squares (1 mm) on a side that are further divided into 16 squares that are 250 µm on a side (c)-the Fuchs-Rosenthal ruling. The 32 1-mm squares hold a total of 0.0064 mL of sample. A sample of 10^4cells/mL will yield *ca*. 64 cells total (two per 1-mm square).

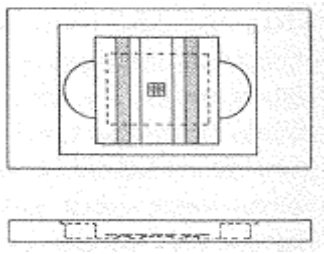
Note that some manufacturers supply slides 0.2 mm deep, but with the Neubauer ruling (d) as described next. These have only 18 1-mm squares in all so that a sample of 10^4 cells/mL yields a total of only 36 cells. Thus, they are less convenient at low cell densities. With all hemacytometers of depth 0.2 mm, algal species larger than *ca*. 75 µm will seldom distribute themselves well, and long, thin species or those forming long colonies will often accumulate near the entry slit or at the chamber edges.

4. Hemacytometer with chambers 0.1 mm Deep



This has two chambers each with a tic-tac-toe arrangement of nine 1-mm squares having several layers of subdivision, including 250-µm squares, 250× 200-µm rectangles, 200-µm squares, and 50-µm squares. This is the Neubauer ruling. The total volume in both ruled chambers (18 squares) is 0.0018 mL; thus, a sample with 10^4 cells/mL yields *ca*. one cell per 1-mm square, 18 cells in all. Counting is best done with 10× or 20× objectives, but standard high dry objectives (40-45×) can be used with no. 1 $^1/_2$ coverslips.

5. Petroff-Hausser Bacteria Counting Slide



This slide has but one chamber, 0.02 mm deep, with improved Neubauer ruling. High dry and oil immersion objectives can be used. Total volume of the ruled area is 0.00018 mL; thus, a sample with 5 × 10^4 cells/mL yields *ca*. one cell per 1-mm square. A sample with 10^6 cells/mL can be tallied, though with some tedium, because the 20 cells (average) within each 1-mm square have to be looked for carefully. For dense cultures, use the central 400 squares of 50-µm sides. A culture of 2 × 10^7 cells/mL yields one cell per 50-µm square (average).

SOP#TP-010
FISH, MEASUREMENT OF LENGTH AND WEIGHT WITH LARVA AND JUVENILE

This SOP is a common procedure for measurement of length and weight with larva and juvenile fish for whole effluent toxicity test. This based on Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 4th Edition, EPA 821-R-02-014, and OECD GUIDELINE FOR TESTING OF CHEMICALS, 212.

STEPS	COMMENTS
1. Materials	
1.1. Organism (Fish)	
1.2. Length measuring board	
1.3. Precision electronic balance (SPG202F, OHAUS)	
1.4. Drying oven (DS-D049, Dong Su Science)	
1.5. Digital Microscopy camera (Moticam 2500, Motic Inc.)	
1.6. Digital measuring length Software (Motic Images Plus 2.0, Motic Inc.)	
1.7. 0.45 μm membrane filter (GFF, WHATMAN)	
1.8. Filter Holders with Receiver (NALGENE)	
1.9. Vacuum pump	
1.10. Aluminum weighing pan	
2. Methods	
2.1. Measuring Length and Wet weight (Juvenile)	
2.1.1. Place a fish on measuring board on its left side, with its head facing the recorder's left.	2.1.1. The fish length will be determined on a measuring board with a linear scale (mm) with a rigid head piece. Before taking measurements of fish, the measuring board will be visually inspected to ensure that the board is in good working order.
2.1.2. Hold the head of the fish firmly against the head piece before measuring the fish	2.1.2 

STEPS	COMMENTS
2.1.3. Measure the total length to the nearest millimeter. Total length is defined as the length from the most anterior part of the fish to the tip of longest	
2.1.4. Note the fish length on the sheet (Condition Index)	
2.1.5. Place a fish next to the Precision electronic balance (SPG202F, OHAUS)	2.1.5. 
2.1.6. When the indication become stable, read the measuring value and note the fish weight on the sheet (Condition Index)	
2.1.7. When end of measuring, packing the all fish by the zipper bag and kept in the freezer.	
2.2. Measuring Length and Dry weight (Larva)	
2.2.1. Filtering the test water of chamber include larva fish with 0.45 μm membrane filter (GFF, WHATMAN).	2.2.1. Using with Vacuum pump, Nalgene Filter Holders with Receiver, Introductions for using. **(SOP#TE-007)**
2.2.2. Transfer filter into aluminum weighing pan	
2.2.3. Place the weighing pan in a Drying oven (DS-D049, Dong Su Science) and set at 60 ℃ during 24 hours.	
2.2.4. After 24 hour, place the membrane filter to the Precision electronic balance (SPG202F, OHAUS)	
2.2.5. When the indication become stable, read the measuring value	
2.2.6. Subtract non used filter’s weight from the drying filter and divide by number of live larva	
2.2.7. Note the Dry weight on the sheet (Condition Index)	
2.2.8. Using the Digital Microscopy camera (Moticam 2500, Motic Inc.) and Digital	

STEPS	COMMENTS
measuring length Software (Motic Images Plus 2.0, Motic Inc.), measuring the total length of larva fish	2.2.8 
2.2.9. Note the total length of larva on the sheet (Condition Index)	

SOP#TP-011
EFFLUENT AND RECEIVING WATER SAMPLING, SAMPLE HANDLING, AND SAMPLE PREPARATION FOR TOXICITY TESTS

This SOP is a common procedure for water sampling , sample handling and preparation. This based on Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters of Freshwater Organisms, 4th Edition, EPA 821-R-02-013.

STEPS	COMMENTS
1. Materials	
1.1. Test water (effluent or receiving water)	
1.2. Water quality checker	
1.3. Water sample container	
1.4. Shipping methods (express, air express, courier service or **refrigerator car**)	
1.5. CP filter (**3㎛**), membrane filter(**0.45㎛**)	
1.6. A water pump, oil pressure hose	
1.7. A cold storehouse (0 – 6)	
2. Methods	
2.1. Effluent sampling	
2.1.1. The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger.	2.1.1. The sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitively large number of separate samples and tests. Collection of 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period.
2.1.2. Considering toxicity test organisms, prepare a container to be able to admit water for need.	2.1.2. Sufficient sample volume must be collected to performed the required toxicity and chemical tests. A 4-L (1-gal) **CUBITAINER®** will provide sufficient sample volume for most tests.
2.1.3. When tests are conducted on-site, test solutions can be renewed daily with freshly.	

STEPS	COMENTS
collected samples, except for the phyto planktons, test which is not renewed.	
2.1.4. When tests are conducted off-site, a minimum of three samples are collected.	2.1.4. If these samples are collected on Test Days 1, 3, and 5, the first sample would be used for test initiation, and for test solution renewal on Days 2. The second sample would be used for test Days 3 and 4. The third sample would be used for test solution renewal on Days 5, 6 and 7.
2.1.5. Aeration during collection and transfer of effluents should be minimized to reduce the loss volatile chemicals.	
2.1.6. Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.	
2.2. Receiving water sampling	
2.2.1. Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity test. Therefore, based on the requirements of test, a single grab sample or daily grab sample of receiving water is collected for use in the test.	
2.2.2. To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge.	
2.2.3. The sampling point is determined by the objectives of the test.	2.2.3. In rivers, samples should be collected from mid-term and at mid-depth, if accessible. In lakes the samples are collected at mid-depth.
2.3. Sample handling	
2.3.1. Unless the samples are used in an on-site toxicity test the day of collection (or hand	2.3.1. Composite samples should be chilled as they are collected. Grab samples should be chilled

STEPS	COMENTS
to the testing laboratory for use on the day of collection), they should be chilled and maintained at **0-6** until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.	and recorded. If the samples are not immediately following collection.
2.3.2. If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.	
2.3.3. EPA believes that **36h** is adequate time to deliver the samples to the laboratories performing the test in most cases.	
2.3.4. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24h, 48h, and/or 72h after first use, if stored at 0-6 , with minimum head space.	
2.3.5. To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.	
2.4. Shipping samples	
2.4.1. Samples collected for off-site toxicity testing are to be chilled to **0-6** during or immediately after collection, and shipped iced to the performing laboratory.	
2.4.2. Several sample shipping options are available, including express, air express, courier	

STEPS	COMENTS
Service **refrigerator car.**	
2.4.3. Upon arrival at the laboratory, samples are logged in and the temperature is measured immediately prepared for testing, they are stored at **0-6** until used.	
2.4.4. Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36h unless a variance has been granted by the NPDES permitting authority.	
2.5. Preparation of water samples for tests	
2.5.1. aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing.	
2.5.2. fish, tests, effluents and receiving waters should be filtered through a **3-㎛ CP-filter** to remove indigenous organisms that may attack or be confused with the test organisms	
2.5.3. Receiving water used in phyto-planktons, zoo-planktons, toxicity tests must be filtered through a **0.45-㎛** pore diameter filter before use.	2.5.3. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm mesh openings to remove debris and/or break up large floating or suspended solids. Because filtration may increase the dissolved oxygen (DO) in the effluent, the DO should be checked both before and after filtering.
2.5.4. The DO concentration in the samples should	

STEPS	COMENTS
be near saturation prior to use.	
2.5.5. Samples may be warmed slowly in open test containers. If DO is still above 100% saturation after warming to test temperature	minutes, using an airstone until the DO is within the prescribed range **(4.0 mg/L)**.
2.5.5. If DO is below **4.0 mg/L** after warming to test temperatures, the solution must be aerated moderately (approximately 500mL/min) for a few samples should be aerated moderately (approximately 500mL/min) for a few minutes using an airstone.	
2.5.6. In static tests (renewal or non-renewal), low DOs mat commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at a rate of 100 bubbles/min. If aeration is necessary, all test solution must be aerated. it is advisable to monitor the DO closely during the first few hours of the test.	2.5.6. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is stared. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.
2.5.7. At a minimum, pH, conductivity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.	

SOP#TP-012
MEASUREMENT OF CHLOROPHYLL *a* AND PHAEOPIGMENTS BY FLUOROMETRIC ANALYSIS

This SOP referred to 'ESS method 150.1' and 'Fluoromter user's manual'.

1.0 Principal

1.1 Chlorophyll *a*, a characteristic algal pigment, constitutes approximately 1% to 2% (dry weight) of planktonic algal biomass. This feature makes chlorophyll *a* a convenient indicator of algal biomass.

1.2 This method is applicable to most surface waters and algal cultures.

2.0 Summary of Method

2.1 Algal cells are concentrated by filtering a known volume of water through a membrane filter (47 mm, 0.45 μm pore size). The pigments are extracted from the concentrated algal sample in an aqueous solution of acetone. The chlorophyll *a* concentration is determined fluorometrically by measuring the absorbance of the extract at 460 nm (excitation filter) and 683 nm (emission filter) wavelengths. The resulting absorbance measurements are then applied to a standard equation.

3.0 Sample Preservation and Preparation

3.1 Chlorophyll *a* samples should be placed in a dark cooler and packed in ice at the time of collection.

3.2 Filter from 10 mL of sample through a 0.45 μm membrane filter, applying vacuum until the sample is dry.

3.2.1 Add 0.2 mL of $MgCO_3$ suspension during the final phase of the filtration.

3.2.2 Fold the filter into quarters, wrap in aluminum foil; place in a desiccator and freeze.

3.2.3 Samples may be held frozen for up to 30 days if taken from waters of pH 7 or greater. Samples from acidic waters should be processed promptly.

4.0 Comments

4.1 Pheophytin, a natural degradation product of chlorophyll, has an absorption peak in the

same spectral region as chlorophyll *a*. It may be necessary to make a correction when pheophytin concentration becomes significantly high.

4.1.1 Corrected chlorophyll *a* refers to the method with the pheophytin correction (acidification method).

4.1.2 Uncorrected chlorophyll *a* refers to the method without the pheophytin correction (Trichromatic method).

4.2 Handle samples in subdued light to prevent photochemical breakdown of the chlorophyll.

4.3 Protect the acetone extract from more than momentary exposure to light.

5.0 Apparatus

5.1 Sonicator cell disruptor, Heat Systems-Ultrasonics Inc., Model W-220F, equipped with a microtip.

5.2 Turner Designs Model 10-AU-005-CE.

5.3 Calibrated 15 mL centrifuge tubes with teflon lined caps.

5.4 Centrifuge capable of attaining 500 g.

5.5 Dark box: Light tight box capable of holding a small test tube rack.

5.6 Standard laboratory glassware including membrane filtration apparatus.

5.7 0.45 µm membrane filters (47 mm).

5.8 Vacuum source.

6.0 Reagents

6.1 Aqueous acetone solution: Mix 90 parts reagent grade acetone with 10 parts Milli-Q water (Millipore Reagent Grade Water System).

6.2 0.1 N Hydrochloric acid: Add 8.3 mL of reagent grade hydrochloric acid and dilute to 1000 mL with Milli-Q water.

6.3 1% Magnesium carbonate suspension: Add 1.0 g of magnesium carbonate powder to 100 mL of Milli-Q water.

7.0 Procedure

7.1 Place the filter containing the concentrated algal sample in a centrifuge tube.

7.1.1 Add about 10 mL of aqueous acetone solution; cap tightly and place in the dark box.

7.2 Repeat Step 7.1 until the desired number of samples have been processed.

7.3 Place the dark box in the 4°C cold room and allow the extract to steep overnight.

7.4 Clarify the extract by centrifuging the extract for 20 minutes at approximately 500 g. (Mix the extract thoroughly before centrifuging.)

7.5 Carefully transfer the clear extract to cuvette and measure the absorbance at 440 nm and 683 nm.

7.6 For corrected samples: Immediately after measuring the absorbance, add 0.1 mL of 0.1 N HCl to the fluorometer cuvette, mix, wait 90 seconds and measure the absorbance specified in Step 7.5.

7.7 Discard the sample, rinse the cell two times with 5 mL portions of aqueous acetone solution.

7.8 Repeat Steps 7.5 through 7.7 until all of the samples have been measured.

SOP# TP-013
ROTIFER, ACUTE TOXICITY TEST

This SOP is based on principle of the ASTM E1440 - 91, Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus* and refers to ROTOXKIT M, Standard Operational Procedure (MicroBioTests Inc.) to assess effects. The SOP is to assess effects chemicals toward Rotifer that using neonates of the rotifer *Brachionus plicatilis* hatched from cyst, an acute toxicity (mortality) test is executed in 24 hours.

DEFINITIONS

Rotifer cyst is that a rotifer embryo arrested at an early stage in development, enclosed in an envelope and resistant to desiccation and temperature extremes. Rotifer cysts are often incorrectly referred to as resting eggs. Upon hydration, embryonic development resumes until a neonate female emerges from the cyst.

Rotifer neonate is a newly hatched, freely swimming rotifer. All neonates hatched from cysts are females.

Test substance is crude soluble chemical substance, effluence and/or receiving water to effect on survival, normality, reproduction or mobility of the test organism.

Test solution is prepared for the test by dissolved test substance on the dilution water and/or natural seawater, by diluted effluence or receiving water on the dilution water and/or natural seawater.

STEPS	COMMENTS
1. Preparation	
1.1 Apparatus 6 well plate, 48 well plate, Pasteur pipet, 10 mL auto-pipet and tips, 1 mL micropipet and tips, Incubator, disposable micropipet, 0.45 μm filter seawater, stereomicroscope (SZ-51, Olympus)	1.1 6 well plate: rinsing vessel 48 well plate: test vessel
1.2 Test Water	
.1 Chemical substance: For making stock solution, the chemical substance is dissolved in the artificial seawater (or natural seawater).	1.2.1 The use of natural seawater might sometimes be desirable.
.2 Effluents and/or receiving seawater: For making stock solution, the effluent or receiving seawater dilute in the natural seawater or artificial seawater using control seawater filtered with 0.45 μm membrane filter.	1.2.2 See SOP#TP-011

STEPS	COMMENTS
1.3 Dilution water Dilution water for the toxicity test is used for artificial seawater or uncontaminated natural seawater (0.45 μm membrane filter).	1.3 Dilution water is used as making concentration of the test solution. The use of natural seawater might sometimes be desirable.
2. Methods	
2.1 Preliminary Test	2.1.1~.2 Preliminary test is range-finding test. For the main test (final definitive test), the concentration range in which effects are likely to occur may be determined on the basis of results from range-finding test. If the test substance is the effluent or receiving water, pass/fail test instead range finding test will be performed. The pass/fail test is determined to 100 % effluent significantly different than the control population.
.1 The concentrations of the test substance range in five concentrations .2 The procedure of preliminary test is equal to section 2.2.1. The control and replicates of each concentration in the preliminary test are not necessary.	
.3 Each test well is checked for died Rotifers at 24 hours after the beginning of the test.	2.1.3 In addition to immobility and mortality, any abnormal behavior or appearance is reported.
2.2 Main Test	
2.2.1 Procedures	
.1 Rotifer cyst hatching is initiated approximately 28~30 h before the beginning of the test.	2.2.1.1 See SOP#TO-007.
.2 Make the 6 concentrations of test solution including control.	2.2.1.2 For the main test at least six concentrations including the control, arranged in a geometric series with a factor not exceeding 3.2, are selected. When a solvent is used to solubilize the test substance, additional control containing the solvent at the same concentration as used in the test culture must be included.
.3 Prepare the two 48 well plates (test vessel) and one 6 well plate (rinsing vessel) labeled test date, concentration and replicate number.	2.2.1.3 Total 12 replicates per test concentration.
.4 Fill with test solution in each well on the rinsing vessel (10 mL) and test vessel (1 mL) using a 10 mL auto-pipet and 1 mL micropipet.	

STEPS	COMMENTS
.5 About 100 newly hatched rotifers are transferred to rinsing well using a disposable micropipet.	2.2.1.5~6 See SOP#TO-001 “2.3 Isolation”. Isolate the test animals aged less than 2 hours under the stereomicroscope. Wait for approximately one hour to allow the rotifers to adapt to the eventual salinity change (e.g. from 20 ppt in the hatching medium to the salinity in the test solution).The volume of medium carried over with the rotifers should be minimized.
.6 Divide 5 animals in each well on test vessel using a Pasteur-pipet to the test animal under the stereomicroscope.	
.7 Cover the cap of the 48 well plate after dividing the test organism.	
.8 A piece of parafilm is stretched across the top of the plate and the cover put on tightly.	
.9 Place the test vessels in the incubator under the test condition.	2.2.1.9 The test condition is showed in Appendix I.
.10 Check and record for died animals at 24 hours after placing the test vessels in the incubator.	2.2.1.10 Stereomicroscope is used for checking the state of test animals. In addition to mortality, any abnormal behavior or appearance is reported. Rotifers are considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.
2.2.2 Measurement of test water	2.2.2 See SOP#TE-006.
Measure the Temperature, DO and pH of the solutions at the beginning and at the end of the test.	But, test vessels contain only 1mL, it is technically difficult to measure DO at the end of the experiment. It is also known that brachionid rotifers are not sensitive to low oxygen levels.

APPENDIX Ⅰ

Test conditions & acceptability criteria

Test parameter	Conditions
Test type	Static (non-renewal)
Temperature	25 ± 1˚C
Light quality	None
Light intensity	Darkness
Photoperiod	0 h light: 24 h dark
Test chamber size	1.5 mL (48 well plate)
Test solution volume	1mL
Renewal of test solutions	None
Age of test organisms	Less than 2 hours
Number of individuals per test chamber	5 inds.
Replicate test chambers per concentration	12
Feeding regime	None
Cleaning	None
Aeration	None
Dilution water	Natural seawater: Filtered seawater (control seawater) by 0.45 μm membrane filter, or Artificial seawater
Test concentration	5 including 100.00% effluents and a control
Dilution factor	Effluents: 0.5
Test duration	24 hours
Endpoints	survival
Test acceptability criteria	90% or greater survival of all control organisms

APPENDIX Ⅱ

Artificial seawater

Composition of artificial seawater (Guillard, R. R. L., 1983)

Component	Stock solution (15 PPT)	Stock solution (34 PPT)
NaCl	11.31 g/L dH_2O	22.62 g/L dH_2O
KCl	0.36 g/L dH_2O	0.83 g/L dH_2O
$CaCl_2$	0.54 g/L dH_2O	1.24 g/L dH_2O
$MgCl.6H_2O$	1.97 g/L dH_2O	4.53 g/L dH_2O
$MgSO_4.7H_2O$	2.39 g/L dH_2O	5.50 g/L dH_2O
$NaHCO_3$	0.17 g/L dH_2O	0.39 g/L dH_2O
* This reconstituted seawater is stirred for 24 h.		

SOP# TP-014
ROTIFER, POPULATION GROWTH TEST

This SOP is based on principle of Cyst-based toxicity tests. VIII. Short-chronic toxicity tests with the freshwater rotifer *Brachionus calyciflorus* (Janssen et al., 1994) and refers to ROTOXKIT F CHRONIC, Standard Operational Procedure (MicroBioTests Inc.) to assess effects. The SOP is to assess chronic effects chemicals that using neonates of the rotifer *Brachionus plicatilis* hatched from cyst, a short-chronic reproduction (population growth) inhibition test is executed in 96 hours.

DEFINITIONS

Rotifer cyst is that a rotifer embryo arrested at an early stage in development, enclosed in an envelope and resistant to desiccation and temperature extremes. Rotifer cysts are often incorrectly referred to as resting eggs. Upon hydration, embryonic development resumes until a neonate female emerges from the cyst.

Rotifer neonate is a newly hatched, freely swimming rotifer. All neonates hatched from cysts are females.

Test substance is crude soluble chemical substance, effluence and/or receiving water to effect on survival, normality, reproduction or mobility of the test organism.

Test solution is prepared for the test by dissolved test substance on the dilution water and/or natural seawater, by diluted effluence or receiving water on the dilution water and/or natural seawater.

STEPS	COMMENTS
1. Preparation	
1.1 Apparatus 6 well plate, 48 well plate, Pasteur pipet, 10 mL auto-pipet and tips, 1 mL micropipet and tips, Incubator, disposable micropipet, 50 mL tube, 0.45 μm filtered seawater, algal stock solution, stereomicroscope (SZ-51, Olympus)	1.1 6 well plate: rinsing vessel 48 well plate: test vessel
1.2 Test Water	
.1 Chemical substance: For making stock solution, the chemical substance is dissolved in the artificial seawater (or natural seawater).	1.2.1 The use of natural seawater might sometimes be desirable.
.2 Effluents and/or receiving seawater: For making stock solution, the effluent or receiving seawater dilute in the natural seawater or artificial seawater using control seawater filtered with 0.45 μm membrane filter.	1.2.2 See SOP#TP-011

STEPS	COMMENTS
1.3 Dilution water Dilution water for the toxicity test is used for artificial seawater or uncontaminated natural seawater (0.45 μm membrane filter).	1.3 Dilution water is used as making concentration of the test solution. The use of natural seawater might sometimes be desirable.
1.4 Algal stock solution	
.1 Take six 50 mL tubes with *Chrorella vulgaris* solution.	
.2 Centrifuge the tubes at 3000 rpm for 10 minutes, pour out the supernatant and add 0.45 μm filtered seawater to 50 mL mark.	
.3 Resuspend the algae and centrifuge again at 3000 rpm for 10 minutes.	
.4 Pour out the rinsing water "to the last drop" and Add filter seawater up to 1 mL mark.	1.4.4 Rinsing water is the supernatant after centrifugation.
.5 Collect a tube with algal suspension.	
2. Methods	
2.1 Preliminary Test The preliminary test is replaced with result of acute toxicity test	
2.2 Main Test	
2.2.1 Procedures	
.1 Rotifer cyst hatching is initiated approximately 28~30 h before the beginning of the test.	2.2.1.1 See SOP#TO-007.
.2 Make the 6 concentration of test solution including control.	2.2.1.2 For the final definitive test at least six concentrations including the control, arranged in a geometric series with a factor not exceeding 3.2, are selected. When a solvent is used to solubilize the test substance, additional control containing the solvent at the same concentration as used in the test culture must be included.
.3 Fill a 15 mL tube with 10 mL filtered seawater and add algal stock solution.	2.2.1.3 Test solution should be contained 1×10^6 cells/mL of *Chlorella vulgaris*.

STEPS	COMMENTS
.4 Add filter seawater up to 15 mL mark and shake to homogenize the test solution.	
.5 Prepare the two 48 well plates (test vessel) and one 6 well plate (rinsing vessel) labeled test date, concentration and replicate number.	2.2.1.5 Total 12 replicates per test concentration.
.6 Fill with test solution in each hole on the rinsing vessel (10 mL) and test vessel (1 mL) using a 10 mL auto-pipet and 1 mL micropipet.	2.2.1.6 * 48 well plate: test solution contained algal * 6 well plate: test solution not contained algal
.7 About 100 newly hatched rotifers are transferred to rinsing well using a disposable micropipet.	2.2.1.7~8 See SOP#TO-001 “2.3 Isolation”.
.8 Divide five animals in each hole on test vessel using a Pasteur-pipet to the test animal under the stereomicroscope.	Isolate the test animals aged less than 2 hours under the stereomicroscope. Wait for approximately one hour to allow the rotifers to adapt to the eventual salinity change (e.g. from 20 PPT in the hatching medium to the salinity in the test solution).The volume of medium carried over with the rotifers should be minimized.
.9 Cover the cap of the 48 well plate after dividing the test organism.	
.10 A piece of parafilm is stretched across the top of the plate and the cover put on tightly.	
.11 Place the test vessels in the incubator under the test condition.	2.2.1.11 The test condition is showed in Appendix I.
.12 Check and record for total number of rotifers every 24 hours after placing the test vessels in the incubator.	2.2.1.12 Stereomicroscope is used for checking the state of test animals. In addition to mortality, any abnormal behavior or appearance is reported. Rotifers are considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.
2.2.2 Measurement of test water Measure the Temperature, DO and pH of the solutions at the beginning and at the end of the test.	2.2.2 See SOP#TE-006. But, test vessels contain only 1 mL, it is technically difficult to measure DO at the end of the experiment. It is also known that brachionid rotifers are not sensitive to low oxygen levels.

STEPS	COMMENTS
2.2.3 Data analysis	
2.2.3.1 Population growth rate	
$r_m = \frac{\ln N_t - \ln N_0}{t}$ ----------[1] Where: r_m is the intrinsic rate of natural increase in the population growth experiment. N_0 and N_t are represented the population size at t=0 and t=4, respectively.	2.2.3.1 Population growth rate is calculated using the equation describing the population growth in an unlimited environment (Krebs, 1985)
2.2.3.2 Percentage growth inhibition	
[2] $I\% = \frac{N_{control} - N_{toxicant}}{N_{control}} \times 100$ ------ where: I% is percent inhibition in average growth rate. N are represented the population in each test concentrations.	2.2.3.2 Calculate the mean number of live rotifers in each test well of the rows containing toxicants.

APPENDIX Ⅰ

Test conditions & acceptability criteria

Test parameter	Conditions
Test type	Static (non-renewal)
Temperature	25 ± 1˚C
Light quality	None
Light intensity	Darkness
Photoperiod	0 h light: 24 h dark
Test chamber size	1.5 mL (48 hole plate)
Test solution volume	1mL
Renewal of test solutions	None
Age of test organisms	Less than 2 hours
Number of individuals per test chamber	5 inds.
Replicate test chambers per concentration	12
Feeding regime	Once at the beginning of the experiment (*Chlorella vulgaris* , 1×10^6 cells/mL)
Cleaning	None
Aeration	None
Dilution water	Filtered seawater (Control seawater) by 0.45 μm membrane filter
Test concentration	5 including 100.00% effluents and a control
Dilution factor	Effluents: 0.5
Test duration	96 hours
Endpoints	population growth rate
Test acceptability criteria	- Population growth rate in the control : ≥ 0.55 - Percentage growth inhibition in the lowest toxicant concentration: < 50%

APPENDIX Ⅱ

Artificial seawater

Composition of artificial sea water (Guillard, R. R. L., 1983)

Component	Stock solution (15 PPT)	Stock solution (34 PPT)
NaCl	11.31 g/L dH_2O	22.62 g/L dH_2O
KCl	0.36 g/L dH_2O	0.83 g/L dH_2O
$CaCl_2$	0.54 g/L dH_2O	1.24 g/L dH_2O
$MgCl{\cdot}6H_2O$	1.97 g/L dH_2O	4.53 g/L dH_2O
$MgSO_4{\cdot}7H_2O$	2.39 g/L dH_2O	5.50 g/L dH_2O
$NaHCO_3$	0.17 g/L dH_2O	0.39 g/L dH_2O
* This reconstituted seawater is stirred for 24 h.		

3. SOP of TO (Test Organisms)

SOP#TO-001
PHYTOPLANKTON, ISOLATION AND CULTURE

The purpose of this SOP is to describe isolation methods of phytoplankton regardless of natural habitat and culture methods of isolated organisms(reference: Algal Culturing TECHNIQUES. 2005. chapter 6)..

STEPS	COMMENTS
1. Materials	
1.1 Collected phytoplankton samples	
1.2 Apparatus 60 ㎛ sieve, 48 well plate, 1mL pipettmen, 1mL pipette tips, isolation tube, Pasteur pipettes, glass spot plate, sterilized test tubes and caps, 250mL Erlenmeyer flasks, torch, f/2 medium, inverted microscope (CKX 31, Olympus)	1.2 Isolation tube with plastic pipette tips at both ends, one serving as a mouthpiece and the other supporting a Pasteur micropipette. Glass spot plate can be used to rinse cells during isolation.
2. Methods	
2.1 Preparation of Micropipette	
2.1.1 The Pasteur pipette is held in the hottest region of the flame, supported on the left by a hand and on the right by forceps.	2.1.1 The most common method is single-cell isolation by micropipette. With minimal practice, this technique becomes quick and easy, but the beginner must spend some timer practicing before reliable production of micropipettes is achieved
2.1.2 When the glass is soft, the pipette is quickly removed from the flame with a gentle pull to produce a thin tube.	
2.1.3 The forceps is then relocated to the appropriate region of the thin area so that it breaks, forming a micropipette.	
2.1.4 The forceps is used to gently bend the thin area so that it break, forming a micropipette.	2.1.4 This tip is not suitable.
2.1.5 An enlarged tip of a micropipette, showing a jagged break.	2.1.5 This tip is suitable for use. That the diameter of the tip is larger than the flagellate cell (bearing microscopic scales), thus reducing the probability of shearing as the cell enters the micropipette during isolation.

STEPS	COMMENTS
2.1.6 An enlarged tip with a very smooth break.	
2.2 Dilution	
2.2.1 An aliquot is removed from the sample jar and placed into a test tube containing sterile medium.	
2.2.2 After mixing, one aliquot is removed from the test tube and dispensed into 48 well plate containing sterile medium, and a second aliquot is removed and added to the middle test tube.	
2.2.3 After mixing, the process is repeated(i.e., dispensed into 48 well plate and added to the test tube on the next).	
2.2.4 Each cycle dilutes the original sample and increases the probability of single-cell isolation	
2.3 Isolation	2.3 This process is repeated until a single algal cell, free of all other protists, can be confidently placed into culture medium. Robust organisms, repeated handling can be achieved without damage; however, for delicate organisms, cell damage is an important concern.
2.3.1 We prepare several micropipettes in advance, whereas others prepare a micropipette immediately before use.	2.3.1 Redrawn pipettes used in seawater often form a small salt crust when any remaining seawater is evaporated.
2.3.2 A flexible, latex tube is attached to a mouthpiece on one end and a micropipette or capillary tube at the other end.	2.3.2 Tubing is usually cut to a longer length, so that one can pass the tubing over the shoulders and around the neck, providing support for comfortable use.
2.3.3 Although various mouthpieces and micropipette connectors can be fitted to the tubing, 1mL plastic pipette tips work well at both ends.	

STEPS	COMMENTS
2.3.4 The operator places the tongue over the mouth piece, places the micropipette tip near the target organism and then removes the tongue to gently allow the capillary action to draw the cell up and into the micropipette tip.	2.3.4 If a dry micropipette is immersed in the sample, then violent capillary action results, drawing substantial unwanted material into the micropipette. After capillary loading with sterile liquid, the micropipette is then directed to the selected cell, and residual capillary action gently draws the cell into the micropipette.
2.3.5 After successful capturing of the cell, the micropipette tip is removed from the sample or droplet; the tip is immersed into the next droplet or 48 well plate; and then by means of gentle blowing into the mouthpiece, the captured cell is discharged into a second, sterile droplet.	2.3.5 The drawing or expelling pressure should be slight, because excessive pressure or rapid movement can damage the cell. Immediately after the cell is discharged into the sterile rinsing droplet and before the cell can settle, it should be picked up again and transferred.
2.3.6 The sterile droplet containing the target cell, and possibly other cells, is then examined microscopically.	
2.3.7 With same technique, a clean micropipette is then used to pick up the cell and transfer it to a third sterile droplet.	
2.3.8 This procedure is repeated only until the single cell is isolated from other cells; unnecessary additional isolation often leads to cell damage.	
2.3.9 After the final capture, the cell is discharged into the final isolation vessel(48 well plate).	

2.4 Culture

2.4.1 Each of isolated organisms is cultured in incubator.

2.4.2 Temperature is 20 .

2.4.3 A light-dark cycle is used continuous light, and intensity of light is 3,000lux.

STEPS	COMMENTS
2.4.4 To achieve more dense growth after isolation is transferred into new sterilized medium of test tube, next to 250mL Erlenmeyer flask (100mL f/2 medium).	
2.4.5 Repeatedly, the culture organisms are incubated under the same condition.	2.4.5 The increment varies with the growth rate of the isolated alga, and careful monitoring is necessary.
2.4.6 Once a dense culture is established, then, subculturing can be made into full-strength medium, provide that sufficient numbers of cells are used as an inoculum.	

SOP#TO-005
TRANSPORTATION AND LABORATORY CULTURING OF FISH

This SOP is a common procedure for transporting and culturing of fish for test. This based on Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters of Freshwater Organisms, 4th Edition, EPA 821-R-02-013, and OECD GUIDELINE FOR TESTING OF CHEMICALS, 203.

STEPS	COMMENTS
1. Materials	
1.1. Test organism (Fish)	
1.2. Dip nets (For lager organism)	
1.3. Wide-bore, smooth glass tube(For larva fish)	
1.4. Holding tank (1000L recirculation tank)	
1.5. Air stone, oxygen compressed gas cylinder or portable pump	
1.6. Plastic bags or large-mouth screw-cap plastic bottles, styrofoam coolers.	
1.7. Food (Live brine shrimp nauplii, *Artemia* or dry food)	
2. Methods	
2.1. Transportation	
2.1.1. Organisms are transported from the base or supply laboratory to a remote test site in culture water in plastic bags or large-mouth screw-cap (500mL) plastic bottles in styrofoam coolers.	
2.1.2. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags or by use of an airstone supplied by a portable pump.	2.1.2. The DO concentration must not fall below 4.0 mg/L.
2.1.3. Upon arrival at the test site, the organisms are transferred to receiving water in holding tank.	2.1.3. If receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning

STEPS	COMENTS
	and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is to be used as the dilution water, caution must be exercised in exposing the test organism to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially to the dilution water. If this group dose not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms may be transferred to the dilution water.
2.2. Holding of fish	
2.2.1. All fish must be obtained and held in the laboratory for at least 12 days before they are used for testing.	
2.2.2. Test fish should not be subjected to changes of more than 3 in water temperature in any 12h period or 2units of PH in any 24-h period.	
2.2.3. Fish should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress.	2.2.3. Dipnets are best for handling lager fish. These nets are commercially available or can be made from small-mesh nylon netting , silk batting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes(4 to 8 mm ID)with rubber bulbs or pipettors should be used for transferring smaller fish such as larval fish.
2.2.4. Holding tanks for fish are supplied with good quality water with flow- through rate of at least two tank volumes per day. Otherwise use a recirculation system where water flows through an activated carbon or undergravel	2.2.4. Maximum loading of 0.1g fish/L for semi-static system is recommended; for flow-through systems higher loading can be accepted. *Holding water : Filtered seawater (3㎛)

STEPS	COMENTS
filter to remove dissolved metabolites.	
2.2.5. Crowding must be avoided because it will stress the fish and lower the DO concentrations to unacceptable levels.	2.2.5. The DO must be maitaned at a minimum of 6.0 mg/L.
2.2.6. The fish should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some fish mortality **(5-10%)** to occur during the first 48h in a holding tank because of individuals that refuse to feed on artificial food and die starvation.	2.2.6. Fish in the holding tanks should generally be fed as in the cultures
2.2.7. Fish should be fed as much as they will eat at least once a day with live brine shrimp nauplii, *Artemia*, or frozen adult brine shrimp, or dry food.	2.2.7. Adult brine shrimp can be supplemented with commercially prepared food such as **TETRAMIN** or **BIORIL** flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.
2.2.8. A daily record of feeding, behavioral observations, and mortality should be maintained.	

SOP#TO-007
ROTIFER, CYST HATCHING METHODS

This SOP is hatching method for rotifer using for the test in laboratory. The procedure described in this SOP is based on principle of the ASTM E1440 - 91, Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus* and refers to ROTOXKIT M, Standard Operational Procedure (MicroBioTests Inc.).

STEPS	COMMENDS
1. Materials	
1.1 Target Organism Cyst of *Brachionus plicatilis*	1.1 Producer: MicroBioTests Inc.
1.2 disposable micropipet, 10 mL auto-pipet and tip, 6 well plate, standard seawater, stereomicroscope (SZ-51, Olympus)	
2. Methods	
2.1 Preparation standard seawater	
.1 Prepare 20 mL hatching medium of 20 PPT by mixing 11.8 mL standard seawater with 8.2 mL deionized water in test tube.	2.1.1 Standard seawater (34 PPT) is artificial seawater (But, the use of natural seawater might sometimes be desirable.). Rotifer cyst hatching has to be carried out in seawater of reduced salinity (20 PPT). Reconstituted seawater may be used for up to seven days, but then it should be discarded.
.2 Fill with 20 PPT hatching medium (2 mL) in one well of 6 well plate using 10 mL auto-pipet.	
2.2 Hatching the rotifer cyst	
.1 Empty the contents of one vial of rotifer cysts into the hatching well of the 6 well plate.	2.2.1 One may rinse the cyst vial with 0.5 mL hatching medium to carry over all the cysts into the hatching well.
.2 Add 8 mL hatching medium to the hatching well in the 6 well plate.	2.2.2 Make sure that most of the cysts are submerged (e.g. by rinsing the sides of the hatching well with a micropipet)
.3 Put a strip of parafilm on the well plate and incubate.	

STEPS	COMMENDS
2.3 Hatching conditions .1 Temperature: 25 ± 1˚C .2 Light source: “cool-white” fluorescent lamp Light cycle: L : D = 24 h :0 h Light intensity: 1,000 ~ 3,000 lux	2.3 Check the cysts hourly after 28 hours incubation to insure collecting the rotifers within 2 hours of hatching. Low temperatures, high salinity, high pH and low light can all delay hatching.

APPENDIX Ⅰ

Artificial seawater

Composition of artificial seawater (Guillard, R. R. L., 1983)

Component	Stock solution (15 PPT)	Stock solution (34 PPT)
NaCl	11.31 g/L dH_2O	22.62 g/L dH_2O
KCl	0.36 g/L dH_2O	0.83 g/L dH_2O
$CaCl_2$	0.54 g/L dH_2O	1.24 g/L dH_2O
$MgCl{\cdot}6H_2O$	1.97 g/L dH_2O	4.53 g/L dH_2O
$MgSO_4{\cdot}7H_2O$	2.39 g/L dH_2O	5.50 g/L dH_2O
$NaHCO_3$	0.17 g/L dH_2O	0.39 g/L dH_2O
* This reconstituted seawater is stirred for 24 h.		

4. SOP of TE (Test Equipments)

SOP # TE-002
WATER QUALITY CHECKER
MODEL WQC-22A (DKK-TOA COPORATION)

This **SOP** is common procedure for using water quality checker (**WQC-22A**). And this **SOP** is based on introduction manual.

STEPS	COMMENTS
1. Materials	
1.1. Indicator Main Body 1.2. Sensor 1Set 1.3. Alkaline Dry Cell LR-14 Type 6Pcs 1.4. Neutral phosphate standard buffer solution pH6.86 100mL 1.5. Phthalic acid chloride standard buffer solution pH4.01 100mL 1.6. Polyethylene beaker 2Pcs 1.7. Electrolyte solution for dissolved oxygen electrode R-5C 1.8. Diaphragm catridge for dissolved oxygen electrode 3Set 1.9. Shoulder belt 1Pc 1.10. Instruction manual 1Copy	
2. Methods	
2.1. Temperature 2.1.1. Sensor into water. 2.1.2. Turning **[ON]** Power switch on the Indicator Main Body. 2.1.3. Read measured value after indication become stable.	
2.2. Dissolved Oxygen 2.2.1. Press **[MODE SELECT]** Key to select measuring item at **"DO"**	2.2.1. It will take about three (3) minutes after turning **[ON]** power switch before the DO electrode become stabilized even when the sensor is kept in connection. with indicator main body.

STEPS	COMMENTS
2.2.2. After indication value become stabilized, read the measuring value. This indication value is compensated by **ATC**.	
2.2.3. In case of minding the fluctuation of the least significant digit of DO measured value, the last digit can be cut by pressing **[LSD ON/OFF]** Key.	
2.2.4. Read the measured value after the indication become stabilized.	
2.3. PH	
2.3.1. Press **[MODE SELECT]** Key to select measuring item at "PH".	
2.3.2. Shack the sensor two or three times towards up and down direction to shake off air bubbles stuck at the tip oh PH electrodes.	2.3.2. If air bubbles are stuck to electrode conductivity cell or between electrodes, this may cause an error in the measurement.
2.3.3. When the indication become stable, read the measuring value.	
2.3.4. Read the temperature at the same time, though **ATC** always works.	
2.3.5. If minding the fluctuation of the measuring value, cut the last digit by pressing **[LSD ON/OFF]** Key.	
2.4. Conductivity measurement	
2.4.1. Press **[MODE SELECT]** Key to select measuring item at **"COND"**.	
2.4.2. Shack the sensor two or three times towards up and down direction to shake off air bubbles completely from electrode.	2.4.2. Please change over the measuring range by pressing [ ] and [ ] (**UP and DOWN** Key) so that correct reading can be done by selection right measuring range. Read the value **"Lo" (Low range)** when the electric conductivity falls on between 0~200 mS/m and read the value **"Hi" (High range)** when the electronic conductivity falls on the range more than 200mS/m **(2mS/cm)**.

STEPS	COMMENTS
2.4.3. When the indication become stable, read the measuring value.	
2.4.4. Read the temperature at the same time though **ATC** to maintains sensitivity's change at **2%/** , always works.	
2.5. Salinity concentration	
2.5.1. Press **[MODE SELECT]** Key to select measuring item at **"COND"**.	
2.5.2. Shack the sensor two or three times towards up and down direction to shake off air bubbles completely from electrode.	
2.5.3. when pressing **[S/m NaCl]** Key once, the measuring unit at indicator display changes to **"NaCl"** and the guide mark " " at the display shifts to the position of **"NaCl"** and the salinity can be read in **"%"** unit.	
2.5.4. As the guide mark " " remains at the last measuring condition even after cutting power, press **[S/m NaCl]** Key again if electric conductivity want to be measured.	
2.5.5. When the indication become stable, read the measuring value.	2.5.5. The electric conductivity is indicated by converting the electric conductivity, all of measurement factors for electric conductivity such as **ATC**, range selection between **"Hi"** and **"Lo"**, **[LSD ON/OFF]** remain working in the same with electric conductivity measurement
2.6. Turbidity	
2.6.1. Press **[MODE SELECT]** Key to select measuring item at **"TURB"**.	

STEPS	COMMENTS
2.6.2. Shack the sensor two or three times towards up and down direction to shake off air bubbles completely from sensor parts..	2.6.2. If air bubbles are stuck to electrode conductivity cell or between electrodes, this may cause an error in the measurement.
2.6.3. When the indication become stable, read the measuring value.	
2.7. After measurement.	
2.7.1. In case that the checker is scheduled to use with in one (1) week, wash electrodes well with clean water or tap water after removing protecting cover then store it in bucket filled with clean water after attaching protecting cover.	2.7.1. **Dissolved oxygen electrode**. In case that the checker is not used for long period, take off the DO electrode from sensor body then wash well the inside of DO electrode after wasting the inner solution. **PH electrodes.** Please cover protection caps onto the tips of PH glass electrode and reference electrode if the sensor is stored for long period. At this time, please fill water in the cap for PH glass electrode but not fill water in the cap for PH reference electrode.

5. SOP of SP (Statistical Procedures)

SOP#SP-101
TOXICITY DATA ANALYSIS WITH TOXCALC SOFTWARE

This SOP is for use ToxCalc software that is a comprehensive statistical application designed for analyzing and reporting dose-response data generated from aquatic and sediment toxicity testing. All statistical analyses follow standard guidelines for acute and chronic toxicity data analysis.

1. Test Setup (chapter 4)

Before you enter raw data, you must first complete initial steps defined as test setup. Test setup involves the choosing of a test type, test name and endpoint, entering sample specific information and defining the test the test groups or concentrations. All of these operations are described in this chapter.

2. Test Types

ToxCalc permits you to select from a variety of test types (e.g., “Larval Fish”, “Bivalve”, etc.), each having a unique set of data fields and endpoints (e.g., “48 hour Survival”, “7 Day Growth”, etc.). Each test *Type* will also have at least one test *Name* which in turn may have a unique set of parameters such as *Title*, *Species*, and *Protocol*. The test *Name* may be any name you assign, but is usually the common name of the test species such as “Inland Silverside” or “Red Abalone”.

ToxCalc provides a number of built-in test types and test names suitable for many common aquatic tests. However, ToxCalc also provides you with all of the necessary tools to modify existing test types or create new test types compatible with most test designs. Procedures for browsing the available test types are described below. Later in this chapter, you will learn how to modify or create new test types, test names, and endpoints.

3. Browsing Test Types

3.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu or choose the *Define Test* toolbar button.

3.2 Select the “Larval Fish” test type by selecting from the test *Type* list box. Depending on the current test name, you will see a new *Title*, *species*, *and Protocol* appear in the Codes group box.

> **Note** The text within each edit box represents a code (left of the hyphen) and a descriptor (right of the hyphen). Only the coded part is stored in the database, while the code-descriptor pair appears on all raw data forms and summary reports.

3.3 Select a different test name such as “Inland Silverside”. You will see the test species change. Repeat this process for the other test names.

3.4 View the list of endpoint types by expanding the *Endpoint* list box. You will see a list of endpoints such as “24 Hr Survival” or “7 Day Growth”. ToxClac will limit all statistical analyses to the chosen endpoint type. For example, if you choose the “Larval Fish” test type, “Inland Silverside” test name and “7 Day Growth” endpoint, ToxClac will specifically analyze the growth data.

Note Some test types will have only one assigned endpoint.

4. Browsing Test Fields

4.1 Perform steps 1-2 as described above under "Browsing Test Types".

4.2 Click on the Fields button to open the Assign Fields dialog box (Figure 4-2).

4.3 Click on the *Next* or *Previous* buttons to move from one test field to another or select from the list box in the lower part of the dialog box.

4.4 When finished browsing the test fields, choose the *Cancel* button. If you choose the *Done* button, any changes will be saved. You will new return to the *Define Test* dialog box.

Very Important Be very careful when modifying the test fields. Data sets already in the database may rely on the original field descriptions when data are retrieved or exported. Although modifying the *Width* or *Format* will have minimal effect, changing the order of the fields or deleting fields can have deleterious effects. You are advised to create a new test type as described later in this chapter if the built-in test types are not compatible with your test design.

5. Browsing Test Endpoints

5.1 Perform steps 1-2 as described above under "Browsing Test Types".

5.2 Click on the Endpoints buttons to move from one endpoint to another if multiple endpoints have been assigned.

5.3 Click on the *Next* or *Previous* buttons to move from one endpoint to another if multiple endpoints have been assigned.

5.4 When finished browsing the *Assign Endpoints* dialog box, choose the *Cancel* button. If you choose the *Done* button, any changes will be saved. You will now return to the *Define Test* dialog box.

Note You should notice that each Endpoint has a corresponding Formula Expression. The formula expression instructs ToxCalc where the raw data are located on the Test Data worksheet and how the endpoint should be calculated. For example, the 24 Hr Survival endpoint of the Larval Fish test type divides the data in the second data column (Day 1) by the first data column (Start), hence the formula expression "[2]/[1]". All references are relative to the Groups column (i.e., the column containing the test concentrations).

Very Important Be very careful when modifying the endpoints. Data sets already in the database may rely on these assignments when the data are retrieved or Total Col can have deleterious effects. You are advised to create a new endpoints as described later in this chapter if the built-in endpoints are not suitable for your test design.

6. Adding New Test Types

6.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu or choose the *Define Test* toolbar button.

6.2 Type a new test type into the *Type* edit box.

6.3 Choose the *Add* button adjacent the *Type* edit box. The entry will be either accepted or rejected if the test already exists.

6.4 The *Assign Fields* dialog box will open. *Type a Field Heading* for the first field. Specify a column *Width* and a column *Format*. The "General" format is the most universal and is recommended for most situations.

Note If you want a field to appear only on the printed raw data form, select the *Use for Setup Only* check box. This is helpful when you want fields for recording data, but have no intention of entering the data or performing calculations based on them.

6.5 Continue by repeating step 4 until all fields have been entered. You can use the *Insert* button to insert a field that was inadvertently left out or choose the *Delete* button to remove a field. All field descriptions will be sequentially listed in the list box near the bottom of the dialog box.

6.6 When finished entering the field descriptions, choose the *Done* button. All field descriptions for the new test type will be saved to the database.

6.7 The *Assign Endpoints* dialog box will open. Type an *Endpoint* such as "24 Hr Survival". Specify the *Total Col* when the data type is quantal or proportional. The entry in the *Total Col* edit box instructs ToxCalc which field contains the "total number" used in calculating a proportion. If the *Total Col* is not properly specified, an error may result in the calculations.

6.8 Build the *Formula Expression* by clicking either one of the *Available Fields* list box entries, one of the *Constructs* buttons, or select one of the *Functions*. To may also use any of the Excel worksheet functions to build the expression. To help you gain an understanding of how the expressions should be constructed, browse the endpoints of some of the built-in test types.

Note If you want the calculated results from the expression to be saved and displayed on the worksheet, construct the expression as follows: Select the field where you want the results to be placed. Then choose the "=" button. Finally build the expressions as you normally would. For example, the expression "[3] = [2]/[1]" will divide data column 2 by data column 1 and place the results in data column 3.

6.9 Select the *Quantal or Proportional Data Type* check box when the expression yields a proportion. With this option selected, ToxCalc sets an upper bounds of one, thus avoiding problems when the expression yields a value greater than unity. If the expression yields a quantitative or continuous variable, de-select this check box.

6.10 Select the *Expected Response is Ascending* check box when the expression yields an ascending variable (i.e., higher concentrations are expected to yield larger variables). For example, if you specify

an expression that yields a proportion dead variable, you would select this option. If the expression yields a descending variable such as “proportion alive” or “mean net weight”, you must de-select this option.

6.11 Continue by repeating steps 7 - 10 until all endpoints are entered. When completed, choose the *Done* button to exit the dialog box and return to the *Define Test* dialog box.

Note At any time you can repeat the procedures above to make revisions to the fields or endpoints.

6.12 Begin assigning a test *Name* by first selecting the appropriate *Title*, *Species*, and *Protocol* from the respective list box. If the desired codes are not already in the list boxes, you must add new codes by selecting the Codes button (see “Editing Codes”).

6.13 Type a name in the Name edit box and choose the Add button. In cases where the entered name already exists, you will be asked to overwrite the existing assignments.

Important Exercise caution when overwriting test names. Data sets already in the database may rely on the original assignments when the data are retrieved or exported.

6.14 Repeat steps 11 – 12 if you want to add additional test names.

6.15 When completed, choose the *Done* button to exit the *Define Test* dialog box and update the *Test Data* worksheet.

7. Deleting Test Types

7.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu.

7.2 Select the test *Type* you want to delete.

7.3 Choose the *Delete* button and confirm the deletion.

Note Deleted test types will no longer appear in the list box, but will remain in the database. Once created, test types can always be undeleted as described below.

8. Undeleting Test Types

8.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu.

8.2 Choose the *Undelete* button. A second dialog box will open which lists all currently deleted test types.

8.3 Select the test type you want to undelete and choose the *Undelete* button. The test type will then be re-inserted into the *Type* list box.

9. Switching Test Types

Switching the test type is one of most common routine operations you will perform when using ToxCalc. This procedure allows you to rapidly change the test type when entering data for a different type of test.

9.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu or choose the *Define Test* toolbar button.

9.2 Choose a test mane from the Type list box.

9.3 Choose a test name from the Name list box.

9.4 Choose an endpoint from the Endpoint list box.

9.5 When completed, choose the *Done* button to exit the *Define Test* dialog box and update the *Test Data* worksheet.

Note You can also select a Test Type directly from the Setup menu without entering the dialogbox.

10. Adding New Test Names

10.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu or choose the *Define Test* toolbar button.

10.2 Choose the appropriate test type from the Type list box.

10.3 Select a *Title*, *Species*, and *Protocol* from the respective list boxes. If the desired code is not already in a list box, you must add the code by choosing the *Codes* button.

10.4. Type a name in the Name edit box and choose the Add button. If the entered name already exists, you will be asked to overwrite the existing assignments.

Important Exercise caution when overwriting test names. Data sets already in the database may rely on the original assignments when data are retrieved or exported.

10.5 When finished, choose the *Done* button to exit the *Define Test* dialog box and update the *Test Data* worksheet.

11. Deleting Test Names

11.1 Open the *Define Test* dialog box by choosing the *Tests* command from the *Setup* menu or choose the *Define Test* toolbar button.

11.2 Choose the test type from the *Type* list box.

11.3 Select the test name you want to delete from the *Name* list box. Choose the *Delete* button and confirm the deletion. The test name will be permanently deleted from the list.

12. Auxiliary Data

ToxCalc permits you to define, store, and recall a set of auxiliary data fields. Thus, if you are entering data for a marine test that requires the monitoring of temperature, pH, dissolved oxygen, and salinity, you can define these fields and they will appear on the Auxiliary Data worksheet. All auxiliary data are summarized on the Summary Report.

13. Browsing Auxiliary Field Sets

13.1 Open the Define Auxiliary dialog box by choosing the Auxiliary command from the Setup menu or choose the Define Auxiliary Fields toolbar buttons.

13.2 Select a name from the list box.

13.3 Choose *Fields* button to open the *Assign Fields* dialog box.

13.4 When done browsing the field descriptions, choose the *Cancel* button. If you choose the *Done* button, ant changes you may have made will be saved. You will return to the *Define Auxiliary* dialog box.

14. Adding Auxiliary Field Sets

14.1 Open the *Define Auxiliary* dialog box by choosing the *Auxiliary* command from the *Setup* menu or choose the *Define Auxiliary Fields* toolbar button.

14.2 Type a name in the *Name* edit box and choose the *Add* button. If the entered name already exists, nothing will happen.

14.3 The *Assign Fields* dialog box will open. Type a *Field Heading* for the first field. Specify a column *Width* and a column *Format*. The “General” format is the most universal and is recommended for most situations.

Note The *Use for Setup Only* option box does not apply to auxiliary data.

14.4 Continue by repeating step 3 until all fields have been entered. You can use the Insert button to insert a field that was inadvertently left out or choose the Delete button to remove a filed. All field descriptions will be sequentially listed in a list box found at the bottom of the dialog box.

14.5 When finished entering the field descriptions, choose the *Done* button. All field descriptions will be saved to the database.

16. Deleting Auxiliary Field Sets

16.1 Open the *Define Auxiliary* dialog box by choosing the *Auxiliary* command from the *Setup* menu or choose the *Define Auxiliary Fields* toolbar button.

16.2 Select a name from the list box.

16.3 Choose the *Delete* button to permanently delete the auxiliary field set.

17. Switching Auxiliary Field Sets

17.1 Open the *Define Auxiliary* dialog box by choosing the *Auxiliary* command from the *Setup* menu or choose the *Define Auxiliary Fields* toolbar button.

17.2 Select a name from the list box.

17.3 Choose the *Done* button to enter the field headings on the *Auxiliary Data* worksheet.

18. Defining a Sample

18.1 Open the *Define Sample* dialog box (Figure 4-5) by choosing the *Sample* command from the *Setup* menu or choose the *Define Sample* toolbar button.

18.2 Enter a Test ID by typing an alphanumeric ID into the *Test* ID edit box. If you specify a number on the left hand side, you can use the spinner button to increment or decrement the ID. For example, if you initially "23GHJ0994", then on a subsequent sample you can press the upper spinner button to change the ID to "24GHJ0994". This field is mandatory and is limited to 10 characters.

18.3 Enter the *Ref Tox ID* corresponding to the *Test ID* of your reference toxicant. This field is optional and is limited to 10 characters.

18.4 Select the appropriate *Sample ID*, *Sample Type*, and *Laboratory ID*. If the codes you need are not available in the lists, choose the *Codes* button to add the required code (see "Editing Codes").

18.5 Enter the *Test Start*, *Test End*, and *Sample* dates using the "mm/dd/yy hh:mm" format (time is optional).

18.6 Select the appropriate concentration units from the *Cond. Units* list box. Alternatively, you can enter custom units directly into the edit box.

18.7 Enter the comments you want attached to the sample. The *Comments* field is limited to 70 characters.

18.8 When finished, choose the *Done* button to exit the *Define Sample* dialog box.

Important ToxClac will overwrite data in the database until you specify a new Test ID, then a new record is created. If you specify a previously assigned Test Id, ToxCalc will maintain duplicate records in the database. However, if you Retrieve a data set and re-analyze the data, duplicate records will be prevented from occurring.

19. Get Last Sample

19.1 Open the *Define Sample* dialog box by choosing the *Sample* command from the *Setup* menu or choose the *Define Sample* toolbar button.

19.2 Choose the *Get Last* button to retrieve the last record in the database. This may be helpful when you startup the program and you want to know the last sample entered.

20. Retrieving a Data Set

20.1 Open the *Define Sample* dialog box by choosing the *Sample* command from the *Setup* menu or choose the *Define Sample* toolbar button.

20.2 Choose the Retrieve button. The Search and Retrieve dialog box will open permitting you to search for any Test ID (see "Searching and Retrieving a Dataset", Chapter 7).

21. Defining Test Groups

ToxCalc supports two methods for entering the test

groups (i.e., test concentrations). One method requires you to enter the individual control names and test groups into a dialog box. The second method does not involve the use of a dialog box, but instead permits you to enter the groups directly onto the worksheet.

22. Assigning Groups-Method 1

22.1 Open the *Assign Groups* dialog box (Figure 4-6) by choosing the *Groups Method 1* command from the *Setup* menu or choose the *Define Groups-Method 1* toolbar button.

22.2 Select the data sheet you want the groups to appear on, either the *Test Data Sheet* or the *Auxiliary Data Sheet*.

22.3 Choose the *Clear Groups*, *Clear Reps* and/or the *Clear All* buttons to clear the previous entries if desired.

22.4 Select the appropriate control names from the dropdown edit boxes or enter the appropriate control name. Use the TAB key to move from one edit box to another. If you want to assign numerical concentrations to the controls, enter the concentration first followed by the name such as "1.5 D-Control". ToxCalc will interpret this as a concentration of 1.5.

Very Important If your test design includes a brine or reagent control, always enter/select the dilution water control from the second dropdown edit box.

22.5 Enter the groups starting at the *Group 1* field low to high. Use the TAB key to move from one field to another.

Note You can also choose the *Get Groups* button to retrieve frequently used test groups. Once you have selected the desired groups, click anywhere outside the GROUPS.XLS workbook to transfer the groups into the dialog box.

22.6 Enter the number of replicates starting at the first control. Use the TAB key to move from one edit box to another. If all groups have the same number of replicates, enter the number of replicates in the edit box just below the *Equal Reps* button. Then choose the *Equal Reps* button.

22.7 You can choose the *Assign Now* button to immediately place the groups on the selected worksheet. You may use this option when you are entering the groups simultaneously on the *Test Data* and *Auxiliary Data* worksheets or if you want to see the changes before you close the dialog box.

22.8 When finished, choose the *Done* button to complete the group assignments.

23. Assigning Groups-Method 2

23.1 Select either the Test Data or Auxiliary Data worksheet.

23.2 Choose the *Groups Method 2* command from the *Setup* menu or choose the *Define Groups-Method 2* toolbar button.

23.3 In the *Groups* column (column 4), select the range of cells you want the first group to appear in.

23.4 Enter the group in the topmost cell of the selected range, then press the RETURN key. The entered group will appear in all cells within the selected range.

23.5 Continue until the last group has been entered, then press the ESC key.

Note Pressing the ESC key is mandatory when you are done entering the groups, otherwise the worksheet will remain in data entry mode and the ToxCalc toolbar will remain hidden.

24. Creating Templates

ToxCalc can create templates for recording test data and/or auxiliary data. You can also randomize the positions of the test containers or identify containers to sample during the course of the test. Once created, the template can be retrieved from the template database. For example, you could create and store the template, conduct the test, then retrieve the template for subsequent data entry and analysis.

24.1 Open the *Templates* dialog box (Figure 4-7) by choosing the *Create Templates* command from the *Setup* menu or choose the *Create Templates* toolbar button.

24.2 If you have not already defined the test groups, sample, test type, and/or auxiliary data, do so by choosing the appropriate button.

24.3 Enter the First Position Number if you want to change the first position number.

24.4 If you want to randomize the position of the test containers, select the *Randomize Test Positions* check box and specify a *Seed* number other than zero. By specifying the same seed number you can duplicate the position assignments in the same order each time.

24.5 If you want the controls to be a part of another data set, select the *Exclude Controls from Positioning* check box.

24.6 Select *Create Worksheet for Test Data* and/or *Create Worksheet for Auxiliary Data* check boxes as needed.

24.7 Select either the *None*, *Normal* or *Normal/Blind* printout options. The *Normal/Blind* option will printout two test data sheets, with and without the test groups indicated.

24.8 When completed, choose the *Create* button. You will receive a printout if specified and the test setup information will be added to the setup database.

25. Editing Codes

ToxCalc permits you to add or modify various codes assigned *Test Title, Species, Protocol, Sample ID, Sample Type, Laboratory ID, Controls, Dilution Water, Brine Reagent,* and *Organism* source. You are initially supplied with all approved codes for the

Toxicity Standardized Electronic Reporting System (TSERF). However, you may have to add your code for the *Laboratory ID*, *Sample ID* (NPDES Permit numbers) for your clients, and vender codes for the *Organism* source. The Test Title codes are independent of the TSERF, therefore you are free to add or delete these codes as necessary.

If you are reporting to the TSERF, you must insure that all modifications to the codes are consistent with the coding scheme specified by the TSERF. If you are certain that you will not be reporting to the TSERF, you can assign any codes you like and modify the coding scheme as appropriate.

26. Adding codes

26.1 Open the *Edit Codes* dialog box (Figure 4-8) by choosing the *Code*s button in the *Define Test*, *Define Sample,* or *Compliance Report* dialog box, or choose the *Codes* command from the *Edit* menu.

26.2 Select the category you want to edit from the List Type list box.

26.3 Enter the code in the *Code* edit box and a short description in the *Description* edit box. You are limited to ten characters for the code, however the TSERF has specific code lengths you must use.

26.4 Choose the *Add* button. The code-descriptor pair is added to the list box at the button of the dialog box.

26.5 Add more codes, change the *List Type* or choose the *Done* button.

Note The text within the lower list box represents a code (left of the hyphen) and a descriptor (right of the hyphen). Only the coded part is stored in the database, while the code-descriptor pair appears on all raw data forms and summary reports.

27. Removing Codes

27.1 Open the *Edit Codes* dialog box as described above if it is not already open.

27.2 Select the code-descriptor from the lower list box.

27.3 Choose the Remove button.

27.4 The code will be removed from the appropriate file.

Caution Remove codes sparingly and only after you are certain they are no longer needed. Data sets already in the database may rely on the original code assignments when data are retrieved or exported.

28. Data Entry

Once you have specified a test type, test name, test groups, and sample information, ToxCalc is prepared to receive raw data. The selected test type determines what type of data can be entered on the *Test Data* worksheet and how the data are processed for any given endpoint.

For instance, if you chose the Larval Fish test type,

you will be entering a start count (i.e., the number of larvae within each test container at the start of the test), the number survived at each of the seven days of the test, the total weight (i.e., weighing pan and larvae), the tare weight (i.e., pan weight only), and the weight count (i.e., weight count (i.e., the number of fish weighed if different from the start count).

29. Entering Test Data

29.1 Activate the *Test Data* worksheet by selecting the *Test Data* worksheet tab.

29.2 If test data already exists on the *Test Data* worksheet, choose the *Clear Data* command from the *Edit* menu or choose the *Clear Data* toolbar button to clear all existing data.

Note If a uniform number will be entered in a column, you can enter a number in the topmost cell and double-click. This will fill the remaining cells with the entered number. This operation is useful when entering the start count or other quantity that is uniform across all replicates.

29.3 If no data are available for a particular cell, enter text such as “LIP” (lost in process) or “NA” (not available). ToxClac will treat text as a null entry. If you leave the cell blank, ToxCalc may assign a zero which is treated differently than a null value.

29.4 If you have entered data for two different controls, make sure the *Two Controls* command under the *Data* menu is checked. If only one control was entered, make sure the command is unchecked.

29.5 The test data are now ready for analysis. If you will be entering auxiliary data (e.g. water quality data), follow the steps outlined below.

30. Entering Auxiliary Data

30.1 Activate the *Auxiliary Data* worksheet by selecting the *Auxiliary Data* worksheet tab.

30.2 If necessary, change the auxiliary data fields as instructed under “Switching Auxiliary Field Sets “, Chapter 4.

30.3 If auxiliary data already exists on the *Auxiliary Data* worksheet, choose the *Clear Data* command from the *Edit* menu or choose the *Clear Data* toolbar button to clear all existing data.

30.4 Enter auxiliary data as appropriate.

30.5 Make sure the *Auxiliary Data* command under the *Data* menu is checked.

30.6 The auxiliary data are now ready for analysis.

31. Double Entry

You can use the double entry procedure when you want to check the accuracy of entered data. After invoking the Double Entry command and entering data a second time, ToxClac compares the two entries and alerts you to any discrepancies.

31.1 After you have completed the data entry on either the *Test Data* or *Auxiliary Data* worksheet,

choose the *Double Entry* command from the *Data* menu or choose the double *Entry* toolbar button.

31.2 Re-enter the data a second time using the RETURN key to move to the next lower cell. When you want to move to another column, use the arrow keys or the mouse.

31.3 If a discrepancy is found between the first and second entry, ToxCalc provides a choice between retaining either the original entry or the second entry.

31.4 When finished entering data, press the ESC key to exit the double entry mode.

32. Sorting Groups

If you created a Template, the test positions may be randomized depending on the options you selected. Once you retrieve the template, you may want to sort the test groups by *Pos* (random order) or by *ID* (sequential order).

32.1 To sort the groups into sequential order, choose the *Sort By ID* command. To re-sort the groups into random order, choose the *Sort By Pos* command from the *Data* menu.

33. Data Analysis

Once all of the raw data has been entered, you are ready to analyze the data. ToxCalc categorizes the main statistical approaches into two main groups. Hypothesis based tests yield a "No Observed Effect Concentration" and a "Lowest Observed Effect Concentration", also known by the acronyms NOEC and LOEC, respectively. The point estimate approach determines concentrations causing a predetermined level of effect (e.g., EC_{50}).ToxCalc can perform both types if analyses sequentially or separately. ToxCalc provides you with a visual depiction of the dose-response relationship to help guide you in selecting the appropriate statistical method.

34. Calculating Hypothesis Based endpoints

34.1 Enter all raw data as described in Chapter 5 and select an *Endpoint* from the *Endpoints* menu.

34.2. Open the *Hypothesis Testing* dialog box (Figure 6-1) by choosing the *Analyze* command from the *Data* menu or choosing the *Analyze Data* toolbar button.

34.3 Each test group will be initially selected in the *Groups* list box if the group mean is greater than zero. You can select and de-select the groups as needed. Use the "down" button to select all groups and the "up" button to de-select all groups.

Note The Groups list box is a multi-select list box, therefore you can toggle a selection by clicking on it.

34.4 Select a statistical method from the *Method* list box. By selecting the *EPA Flowchart* method, EPA recommended data analysis schemes are applied (see Appendix C).

34.5 Select either a one-tailed or two-tailed test

from the *Tails* list box. Select the one-tailed option when you expect the sample to exhibit either a positive or negative effect. Select the two-tailed option when the direction of the effect is unknown.

34.6 Select al data transform from the *Data Transform* list box. If the *Auto* checkbox is checked, ToxCalc will automatically select the Arcsin Square Root transform for proportional/quantal data and the no transform for continuous/quantitative data. If you select a transform that requires variables, enter the variables in the appropriates edit boxes.

34.7 Choose the *Alphas* button when you want to change the alpha level (i.e., type I error rate). By default, equality of variance and normality assumptions are tested at 0.01, while the hypothesis test and control comparison are tested at 0.05.

34.8 Choose the *Calculate* button to analyze the data.

34.9 If two controls were entered, choose the appropriate control option when the *Control Options* dialog box opens.

34.10 The *Hypothesis Testing* dialog box will expand and display a dose-response plot of the data including ranges if more than one replicate was entered.

34.11 Review the results in the *Results* list box and determine whether to proceed to point estimation by choosing the *Point Estimates* button or re-analyze the data by changing the selected groups, statistical method or data transform.

34.12 If you do not need to analyze the data set for point estimates, you can choose the Save Data button to save the summary and raw data, as well as create a summary report. Choose the Don't Save button when you want to create a summary report, but you don't want to save the data to the database. If you press the ESC key, all processes will be aborted.

Important Once you have chosen the Save Data button, all raw and summary data will be written to the database. If you have re-analyzed a data set and did not change the endpoint, all raw and summary data from the previous analysis will be overwritten.

35. Calculating Point Estimates

35.1 Complete steps 1-11 as described above.

35.2 Open the Point Estimate dialog box (Figure 6-2) by choosing the Point Estimate button in the Hypothesis Testing dialog box.

35.3 All test groups will be initially selected in the *Groups* list box. You can select and de-select the groups as needed. Use the "down" button to select all groups and the "up" button to de-select all groups.

Note The Groups list box is a multi-select list box, therefore you can toggle a selection by clicking on it.

35.4 Select a point estimate method from the Method list box. If the Auto checkbox is checked,

ToxCalc will automatically select the *Maximum Likelihood-Probit* method for proportional/quantal data and the Linear *Interpolation* (*ICp*) method for continuous /quantitative data.

35.5 Choose the *Options* buttons if you want to change specific options.

35.6 Choose the *Calculate* button to analyze the data.

35.7 The Point Estimate dialog box will expand and display a dose-response plot of the data including confidence limits if available.

35.8 Review the results in the Results list box and determine whether or not to re-analyze the data by changing the point estimate method or the selected groups.

35.9 Choose the Save Data button to save the summary and raw data, as well as create a summary report. Choose the Don't Save button when you want to create a summary report, but you don't want to save the data to the database. If you press the ESC key, all processes will be aborted.

Important Once you have chosen the *Save Data* button, all raw and summary data will be written to the database. If you have re-analyzed a data set and did not change the endpoint, all raw and summary data from the previous analysis will be overwritten.

36. Control Options

Various options are available when calculating point estimates depending on the method you are using. When using any of the maximum likelihood procedures or the Spearman-Karber method, you can choose to assign no response to the control, use the empirical control data, or base the control response on a theoretical lower threshold which you specify.

36.1 After you have selected the point estimate *Method*, but before you choose the *Calculate* button, choose the *Options* button to open the *Control Options* dialog box (Figure 6-3).

36.2 Select the appropriate option. If you select the Theoretical Lower Threshold option, specify the response as a proportion.

36.3 Choose the OK button and calculate the data set.

37. Interpolation Option

When using either the linear or non-linear interpolation procedures, you can specify the ICp, the number of bootstrap resamples, the random sampler seed number, whether the 95% confidence limits are based on the 2.5% and 97.5% quantiles or the ICp ± 2 SD, and whether you want to expand the confidence limits when less than 7 replicates are used. If you choose the Non-Linear Interpolation method, you can also choose to transform the dose (log) and/or the response (log or logit).

37.1 After you have selected the point estimate Method, but before you choose the Calculate button, choose the Option button to open the Interpolation

Options dialog box (Figure 6-4).

Note If you selected the Non-Linear Interpolation method, the Interpolation Options dialog box will be as shown in Figure 6-4. If you selected the Linear Interpolation method, the transform group box will not be shown.

37.2 Select the appropriate options as needed. When selecting the ICp endpoints, hold down the shift key while making a multiple selection.

37.3 Choose the OK button and calculate the data set.

SOP#SP-102
LINEAR REGRESSION ANALYSIS WITH EXCEL PROGRAM

This SOP is for use Excel program that is general statistical application designed for linear regression analyzing between chl-*a* and cell density in the control. The principal for linear regression was applied from 'Linear regression' in BIOMETRY (Sokal and Rohlf, 1981)

Analysis Step

1. Input the information of test on the cell of Excel program as follow

1.1 COC Code

1.2 Test date (Start and end of the test date)

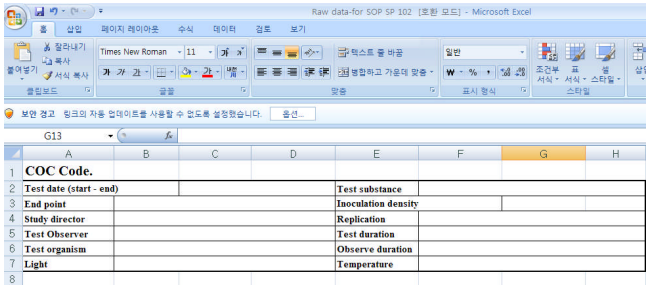
1.3 Test substance, End point, Inoculation density

1.4 Study director, Test observer

1.5 Replication, Test duration, Observe duration

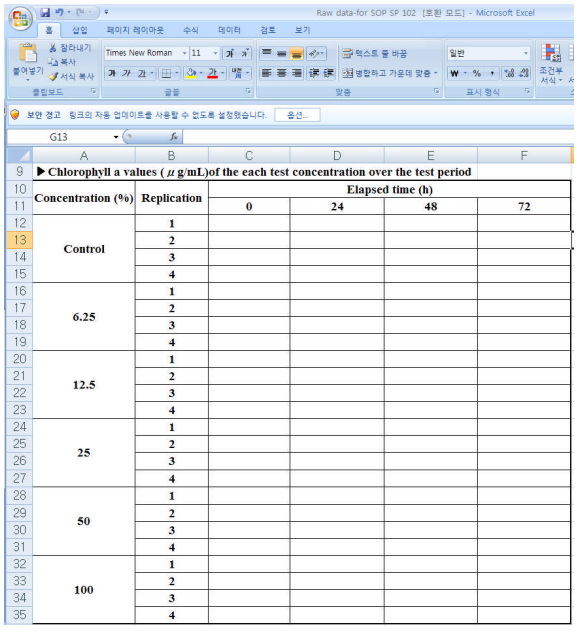
1.6 Test organism

1.7 Light and temperature conditions



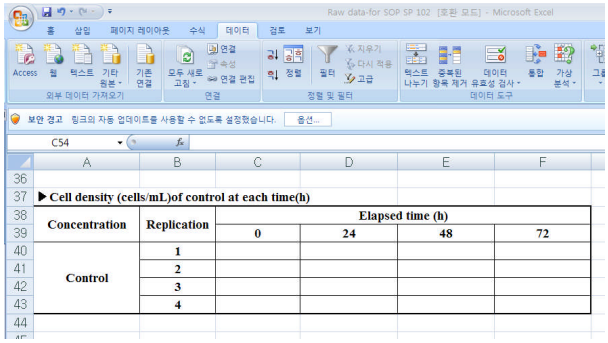
1	**COC Code.**						
2	**Test date (start - end)**			**Test substance**			
3	**End point**			**Inoculation density**			
4	**Study director**			**Replication**			
5	**Test Observer**			**Test duration**			
6	**Test organism**			**Observe duration**			
7	**Light**			**Temperature**			

2. Input the Chlorophyll *a* values of the each test concentration



9	▶ **Chlorophyll a values (*μ* g/mL)of the each test concentration over the test period**					
10	**Concentration (%)**	**Replication**	**Elapsed time (h)**			
11			**0**	**24**	**48**	**72**
12	**Control**	**1**				
13		**2**				
14		**3**				
15		**4**				
16	**6.25**	**1**				
17		**2**				
18		**3**				
19		**4**				
20	**12.5**	**1**				
21		**2**				
22		**3**				
23		**4**				
24	**25**	**1**				
25		**2**				
26		**3**				
27		**4**				
28	**50**	**1**				
29		**2**				
30		**3**				
31		**4**				
32	**100**	**1**				
33		**2**				
34		**3**				
35		**4**				

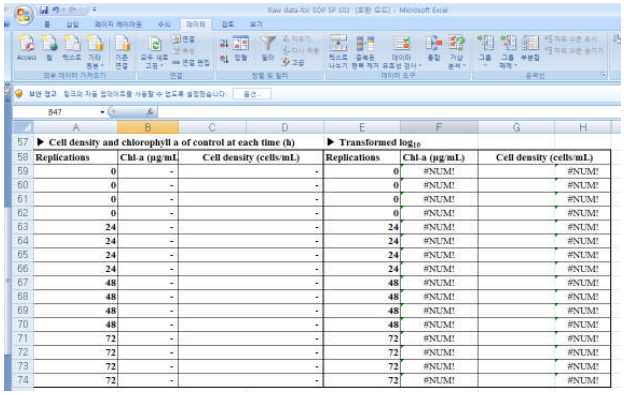
3. Input the cell density of control



36						
37	▶ **Cell density (cells/mL)of control at each time(h)**					
38	**Concentration**	**Replication**	**Elapsed time (h)**			
39			**0**	**24**	**48**	**72**
40	**Control**	**1**				
41		**2**				
42		**3**				
43		**4**				
44						

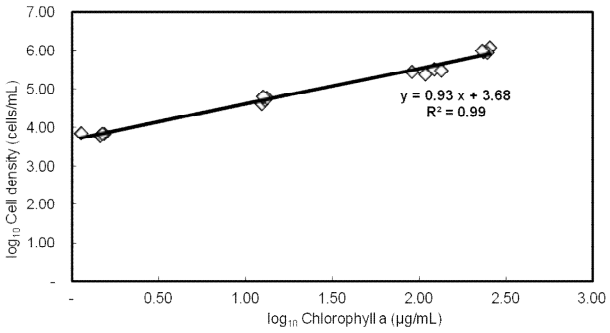
4. Cell density and chlorophyll a of control at each time is formed into line as follow figure

5. Cell density and chlorophyll a values is automatically transformed as $\log_{10}$ value (command in Excel: =LOG10 (Excel cell number of cell density or chlorophyll a value))



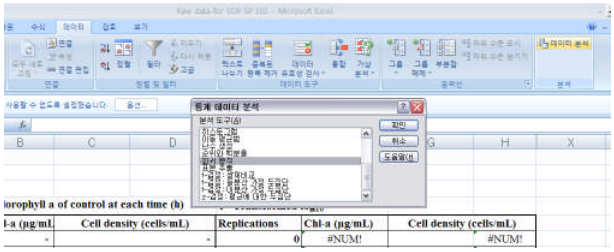
57	▶ **Cell density and chlorophyll a of control at each time (h)**			▶ **Transformed $\log_{10}$**		
58	**Replications**	**Chl-a (μg/mL)**	**Cell density (cells/mL)**	**Replications**	**Chl-a (μg/mL)**	**Cell density (cells/mL)**
59	0	-	-	0	#NUM!	#NUM!
60	0	-	-	0	#NUM!	#NUM!
61	0	-	-	0	#NUM!	#NUM!
62	0	-	-	0	#NUM!	#NUM!
63	24	-	-	24	#NUM!	#NUM!
64	24	-	-	24	#NUM!	#NUM!
65	24	-	-	24	#NUM!	#NUM!
66	24	-	-	24	#NUM!	#NUM!
67	48	-	-	48	#NUM!	#NUM!
68	48	-	-	48	#NUM!	#NUM!
69	48	-	-	48	#NUM!	#NUM!
70	48	-	-	48	#NUM!	#NUM!
71	72	-	-	72	#NUM!	#NUM!
72	72	-	-	72	#NUM!	#NUM!
73	72	-	-	72	#NUM!	#NUM!
74	72	-	-	72	#NUM!	#NUM!

6. The data of chlorophyll a and cell density transformed $\log_{10}$ value are plotted on the graph and equation that is functional dependence of Cell density (Y) on chlorophyll *a* (X) is also represented

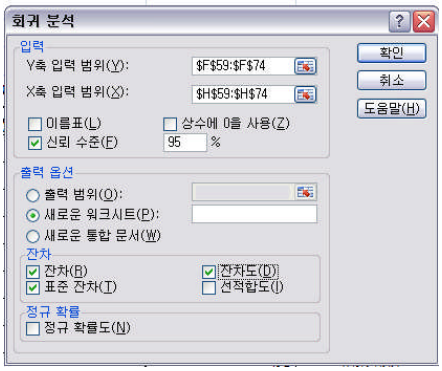


7. The relationship depicted in above graph can be expressed by the formula Y=*b*X+*a*. Therefore, check the coefficient *b* (a slop factor), R^2 value (coefficient of determination) and significant level (*p* value) as follow

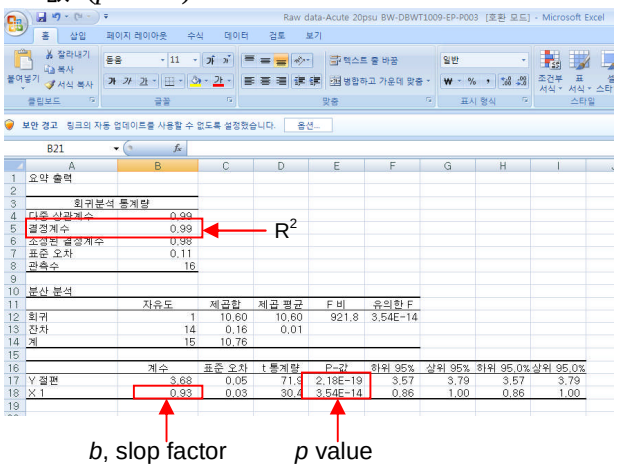
7.1 Click the "데이터 (Data)" tap and "데이터분석 (data analysis)"



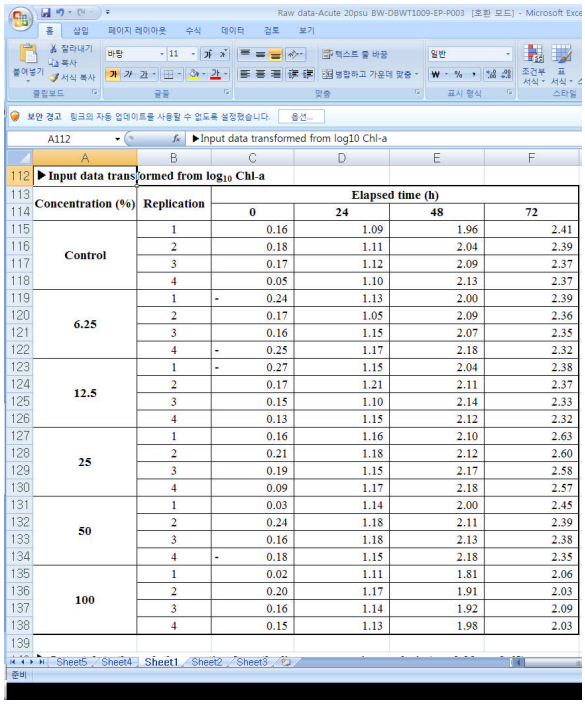
7.2 Choice the 회귀분석(regression analysis). And Select "Y축 입력범위 (dependent variable: transformed cell density)" and "X축 입력범위 (independent variable: transformed chlorophyll *a*)" axis range. And check "신뢰수준 (the confidence level)" as 95%. And click the "확인 (OK)"



7.3 Check the "계수 (*b*: a slop factor)", "결정계수 (R^2 value: coefficient of determination)" and "P-값 (*p* value)"

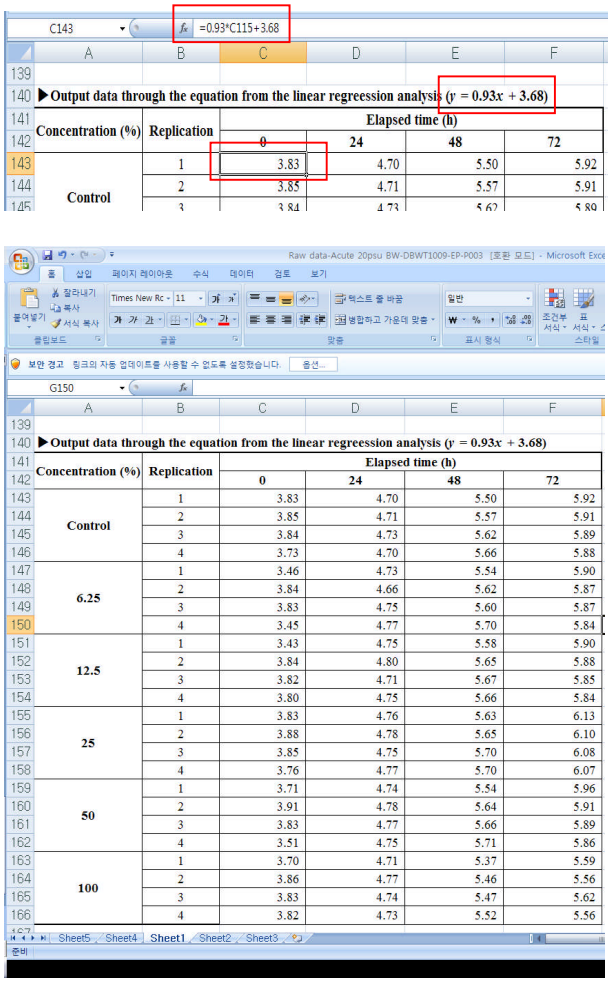


8. Input data transformed log10 chlorophyll-a



Input data transformed from log10 Chl-a					
Concentration (%)	Replication	Elapsed time (h)			
		0	24	48	72
Control	1	0.16	1.09	1.96	2.41
	2	0.18	1.11	2.04	2.39
	3	0.17	1.12	2.09	2.37
	4	0.05	1.10	2.13	2.37
6.25	1	-0.24	1.13	2.00	2.39
	2	0.17	1.05	2.09	2.36
	3	0.16	1.15	2.07	2.35
	4	-0.25	1.17	2.18	2.32
12.5	1	-0.27	1.15	2.04	2.38
	2	0.17	1.21	2.11	2.37
	3	0.15	1.10	2.14	2.33
	4	0.13	1.15	2.12	2.32
25	1	0.16	1.16	2.10	2.63
	2	0.21	1.18	2.12	2.60
	3	0.19	1.15	2.17	2.58
	4	0.09	1.17	2.18	2.57
50	1	0.03	1.14	2.00	2.45
	2	0.24	1.18	2.11	2.39
	3	0.16	1.18	2.13	2.38
	4	-0.18	1.15	2.18	2.35
100	1	0.02	1.11	1.81	2.06
	2	0.20	1.17	1.91	2.03
	3	0.16	1.14	1.92	2.09
	4	0.15	1.13	1.98	2.03

9. For the estimation of cell density at each test concentration, input the equation from linear regression analysis. And these data are transformed $\log_{10}$ cell density

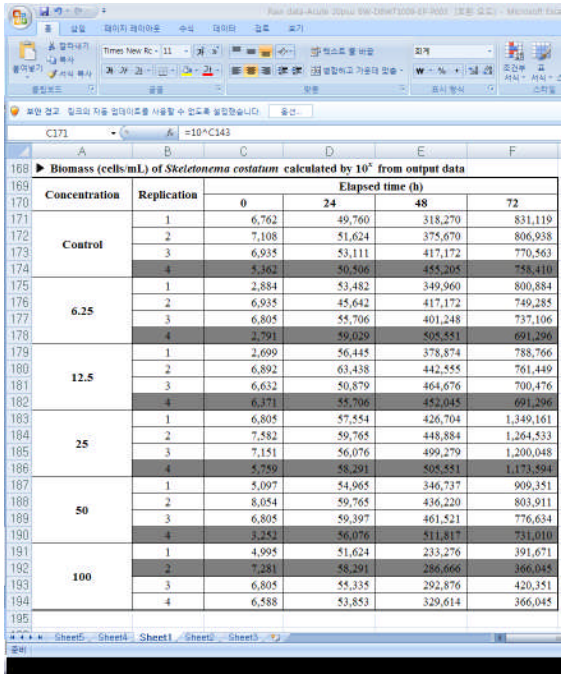


C143 =0.93*C115+3.68

▶ Output data through the equation from the linear regression analysis ($y = 0.93x + 3.68$)

Concentration (%)	Replication	Elapsed time (h) 0	24	48	72
Control	1	3.83	4.70	5.50	5.92
	2	3.85	4.71	5.57	5.91
	3	3.84	4.73	5.62	5.89
	4	3.73	4.70	5.66	5.88
6.25	1	3.46	4.73	5.54	5.90
	2	3.84	4.66	5.62	5.87
	3	3.83	4.75	5.60	5.87
	4	3.45	4.77	5.70	5.84
12.5	1	3.43	4.75	5.58	5.90
	2	3.84	4.80	5.65	5.88
	3	3.82	4.71	5.67	5.85
	4	3.80	4.75	5.66	5.84
25	1	3.83	4.76	5.63	6.13
	2	3.88	4.78	5.65	6.10
	3	3.85	4.75	5.70	6.08
	4	3.76	4.77	5.70	6.07
50	1	3.71	4.74	5.54	5.96
	2	3.91	4.78	5.64	5.91
	3	3.83	4.77	5.66	5.89
	4	3.51	4.75	5.71	5.86
100	1	3.70	4.71	5.37	5.59
	2	3.86	4.77	5.46	5.56
	3	3.83	4.74	5.47	5.62
	4	3.82	4.73	5.52	5.56

10. Re-transform to 10^x the cell density of output data from the equation of regression analysis (command in Excel: 10^ C143 (Excel cell number of output data))



C171 =10^C143

▶ Biomass (cells/mL) of *Skeletonema costatum* calculated by 10^x from output data

Concentration	Replication	Elapsed time (h) 0	24	48	72
Control	1	6,762	49,760	318,270	831,119
	2	7,108	51,624	375,670	806,938
	3	6,935	53,111	417,172	770,563
	4	5,362	50,506	455,205	758,410
6.25	1	2,884	53,482	349,960	800,884
	2	6,935	45,642	417,172	749,285
	3	6,805	55,706	401,248	737,106
	4	2,791	59,029	505,551	691,296
12.5	1	2,699	56,445	378,874	788,766
	2	6,892	63,438	442,555	761,449
	3	6,632	50,879	464,676	700,476
	4	6,371	55,706	452,045	691,296
25	1	6,805	57,554	426,704	1,349,161
	2	7,582	59,765	448,884	1,264,533
	3	7,151	56,076	499,279	1,200,048
	4	5,759	58,291	505,551	1,173,594
50	1	5,097	54,965	346,737	909,351
	2	8,054	59,765	436,220	803,911
	3	6,805	59,397	461,521	776,634
	4	3,252	56,076	511,817	731,010
100	1	4,995	51,624	233,276	391,671
	2	7,281	58,291	286,666	366,045
	3	6,805	55,335	292,876	420,351
	4	6,588	53,853	329,614	366,045

Reference

Sokal, R.R and F. J. Rohlf. 1981. Linear regression. In: Biometry. W.H. Freeman and company, San Francisco, U.S.A., 454-560.

Operation Procedures and Chain Of Custody (COC)

for

Kwang San Co., Ltd.

Appendix 3.4.4

Operation Procedures and Chain Of Custody (COC)

1. Operation Procedures and Chain Of Custody (COC)
   Check List for BWMS Land-based Test
2. Failure and Repair Report
3. Inspection Report
4. Operation Report
5. Seal Paper

CHECK LIST FOR BWMS LAND-BASED TEST

MODEL	TITLE	VOLUME
BioVioletTM	BALLAST WATER MANAGEMENT SYSTEM	250m^3/hr

KWANG SAN _____

Inspecting Officer KOMERI _____

Co-Organization KTR (Chemical analysis) _____

Co-Organization KTR (Corrosion test) _____

Co-Organization MEI _____

KWANG SAN Research & Development Center

Sheet NO.

PART LIST

	Device	Q'YT	Remarks	Responsibility
1	Test Water Tank	3		Kwarg San
2	Ballast Pump	1		
3	Flowmeter	1		
4	Pressure meter	2		
5	Tank Temperature Sensor	9		
6	Level Sensor	3		
7	Filter	1		
8	UV Chamber	1		

MAKING Test Water

Date : . . . ~ . .

	Task	Time	Remarks	Responsibility
1	Tank1 Fresh Water		m/ m³	Kwarg San
2	Tank2 Fresh Water		m/ m³	
3	Tank1 Seawater		m/ m³	
4	Tank2 Seawater		m/ m³	

Date : . .

	Task		Time	Remarks	Responsibility
1	Test Water Drain				Kwarg San
2	Agitator1 "ON"				
3	Agitator2 "ON"				
4	Tank1	Additives make-up (DOC,POC,TSS)		Glucose: kg	
				Starch: kg	
				Silica: kg	
		H-Bacteria		ℓ	KOMERI
		Plankton ≥50㎛		ℓ	PKNU
		Plankton ≥10~50㎛		m³	
5	Tank2	Additives make-up (DOC,POC,TSS)		Glucose: kg	Kwarg San
				Starch: kg	
				Silica: kg	
		H-Bacteria		ℓ	KOMERI
		Plankton ≥50㎛		ℓ	PKNU
		Plankton ≥10~50㎛		m³	
6	Check homogenization				KOMERI
7	Sampling for Test water				
8	Tank1 level			m/ m³	Kwarg San
9	Tank2 level			m/ m³	
10	Check Flowmeter				
11	Check Pressure meter				

Date : . .

BALLASTING (TREATED MODE)

	Task		Time	Remarks	Responsibility
1	Auto Control mode ☐ UV intensity: mW/cm² ☐ Lamp Power: kW				■ Kwang San
2	Turn "OPEN" valves (Pre-Treatment mode) / Lamp Warming-up				
3	Turn "ON" Ballast Pump (Treatment mode) (Flow rate: 250m³/hr)				
4	Sampling for Treated water	Beginning			■ KOMERI ☐ KTR
		Middle			
		End			
5	Agitator2 "OFF"				■ Kwang San
6	Programmed Backflushing				
7	Finish Treated mode				

BALLASTING (CONTROL MODE)

	Task		Time	Remarks	Responsibility
1	Check homogenization				KOMERI
2	Turn "OPEN" valves (Pre-Control mode)				■ Kwang San
3	Turn "ON" Ballast Pump (Control mode) (Flow rate: 250m³/hr)				
4	Sampling for Control water	Beginning			■ KOMERI ☐ KTR
		Middle			
		End			
5	Agitator1 "OFF"				■ Kwang San
6	Finish Control mode				

Date : . .

	Task	Time	Remarks	Responsibility
1	Agitator2 "ON"			■ Kwang San
2	Agitator3 "ON"			

DE-BALLASTING (TREATED MODE)

	Task		Time	Remarks	Responsibility
1	Check homogenization				■ KOMERI
2	Auto Control mode ☐ UV intensity: mW/cm² ☐ Lamp Power: kW				■ Kwang San
3	Turn "OPEN" valves (Pre-Treatment mode) / Lamp Warming-up				
4	Turn "ON" Ballast Pump (Treatment mode) (Flow rate: 250m³/hr)				
5	Sampling for Treated water	Beginning			■ KOMERI ☐ KTR ☐ MEI
		Middle			
		End			
6	Agitator3 "OFF"				■ Kwang San
7	Finish Treated mode				

DE-BALLASTING (CONTROL MODE)

	Task		Time	Remarks	Responsibility
1	Check homogenization				■ KOMERI
2	Turn "OPEN" valves (Pre-Control mode)				■ Kwang San
	Turn "ON" Ballast Pump (Control mode) (Flow rate: 250m³/hr)				
3	Sampling for Control water	Beginning			■ KOMERI ☐ KTR ☐ MEI
		Middle			
		End			
4	Agitator2 "OFF"				■ Kwang San
5	Finish Control mode				

Remarks	

CHECK BY		WITNESS BY	
KWANG SAN		KOMERI	
		KTR	
		MEI	

2. Failure and Repair Report

Failure and Repair Report		Report No.	
		Report Date	
		Reported by	
1	Detection		
2	Component		
3	Operating condition (MODE)		
4	Description		
5	Cause		
6	Repair time		
7	Description of repair action		
Remarks			

Recorded by	KWANG SAN
Confirmed by	KOMERI

3. Inspection Report

Inspection Report		Report No.	
		Report Date	
		Recorded by	
Inspection description			
Inspection component			
Description of inspection action			
No.	Work	Responsibility	Remark
1		KWANG SAN	
2			
3			
4			
Inspection time			
Remarks			

Recorded by	KWANG SAN
Confirmed by	KOMERI

4. Operation Report

Operation Report	Report No.	
	Report Date	
	Recorded by	

NOTICE	All unregistered activities without permission of KOMERI in LBTU MUST BE REPORTED in this written form.

REASON of activity

Preparation	CLEANING		Maintenance	INSPECTION	
	SEA WATER			FAILURE AND REPAIR	
	BRACKISH WATER				

Cleaning procedure

No.	Work	Time	Value	Responsibility	Remark
1				KWANG SAN	
2					
3					
4					
5					

Brackish water and sea water

No.	Work	Time	Value	Responsibility	Remark
1				KWANG SAN	
2					
3					
4					
5					
6					

Related reports

No.	Checklist	Related Report No.	Responsibility	Remark
1			KWANG SAN	
2				
3				

Remarks	

Recorded by	KWANG SAN
Confirmed by	KOMERI

5. Seal Paper

Supervised by: _____ sign.
Date: _____
Place: _____
No.: _____ ser no. 001

Supervised by: _____ sign.
Date: _____
Place _____
No. _____ ser no. 002

Supervised by: _____ sign.
Date: _____
Place: _____
No.: _____ ser no. 003

Supervised by: _____ sign.
Date: _____
Place _____
No. _____ ser no. 004